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THE ROLE OF GLUTAMATE-PYRUVATE TRANSAMINATION IN REPLETION OF CITRIC ACID CYCLE INTERMEDIATES DURING ELECTRICAL STIMULATION OF ISOLATED, IN SITU DOG GASTROCNEMIUS MUSCLE

by

© GARY W. NESS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The Role of Glutamate-Pyruvate Transamination in Repletion of Citric Acid Cycle Intermediates During Electrical Stimulation of Isolated, In Situ Dog Gastrocnemius Muscle", submitted by Gary W. Ness in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physical Education.

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DEDICATION

To my wife, Jan, for her companionship and support during my time as a graduate student.

ABSTRACT

Coupled transaminations involving glutamate-pyruvate transaminase (GPT) in providing for citric acid cycle (CAC) flux and CAC concentration changes during muscular exercise was examined. Varied trains of electrical impulses, delivered directly to isolated, in situ dog gastrocnemius muscles, were used in producing two levels of submaximal metabolic activity. Blood and muscle tissue sample intervals were selected to correspond to the steady state exercise conditions. Arterial-venous differences for glucose, lactate, pyruvate, alanine and lysine, plus muscle concentrations of glycogen, alanine and lysine were used to estimate the percentage of carbohydrates used for oxidative energy supply, $\%\dot{V}O_2(CHO)$, the rate of production of αketoglutarate (αKG) by GPT and the flux rates of the malate-aspartate shuttle and CAC.

Estimates of $\%\dot{V}O_2(CHO)$ revealed that under resting conditions, 10-14% of the total energy supply of the muscle was from carbohydrate. In exercise, similar estimates showed that carbohydrates provided 58-76% and 44-62% of the substrate for "mildly" and "severely" stimulated muscles, respectively. αKG from coupled transaminations involving GPT was estimated to account for 1-5% of the oxidative energy needs of resting muscle whereas during both intensities of exercise, this source accounts for 7-12% of the oxidative demands of the muscle. The absolute quantities of αKG generated from GPT and coupled transiminations was found to be small, in the order of 0.003 - 0.017 μM of αKG/g/min at rest and 0.111 - 0.230 μM of αKG/g/min during both intensities of exercise. These data suggested that only small concentration changes in the CAC intermediate pool of skeletal muscle could be associated with this repletive mechanism during steady state submaximal exercise.

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INTRODUCTION

Research into the physiology and biochemistry of exercise over the past several decades has served to focus attention closely on the muscle cell. Accumulating data have led logically to questions of the biochemical relationships responsible for the physiological properties of the muscle cell, particularly in light of the identification of muscle fiber types. The relationship existing between cell type and metabolic factors has been highlighted by the correlation of the anatomical and functional requirements of a muscle, the relative occurrence of specific fiber types in the muscle, and the recruitment patterns of fiber types during muscular activity (Close, 1972; Gollnick et al., 1972b; Burke and Edgerton, 1975; Edgerton, 1976).

It is now commonly accepted that mammalian muscle is composed of cells which differ significantly in contraction time, peak tension development, fatigue resistance and primary metabolic pathways for energy production (Close, 1972; Ariano et al., 1973; Burke and Edgerton, 1975). Although knowledge of the interplay of speed, force and duration requirements in influencing recruitment patterns of specific fiber types is incomplete, present data (Gollnick et al., 1973c, d; 1974a, b; 1975; Edgerton et al., 1975, Wenger and Reed, 1976) suggests that high intensity, short duration exercise requires substantial recruitment of fast twitch, readily fatiguable fibers. On the other hand, prolonged submaximal muscular activity relies upon the sustained recruitment of the fatigue resistant pool of fibers.

The physiological phenomenon of fatigue resistance depends specifically on the metabolic pathways which supply energy to the muscle cell (Close, 1972; Burke and Edgerton, 1975, Wenger and Reed, 1976). Muscle fibers which rely predominantly on glycolysis for energy production are rapidly fatigued. High intensity exercise requires a high rate of energy production and glycolysis is geared to meet this demand (Lehninger, 1970; Keul et al., 1972). However, glycolysis alone, releases only a small fraction of the chemical energy available in the glucose molecule (Keul et al., 1972). Lactic acid is almost as complex a molecule as glucose since its carbon atoms remain in the same oxidation state, retaining on the average the same number of hydrogen atoms per carbon (Lehninger, 1970). Moreover, lactic acid accumulation in the muscle cell may directly inhibit the contractile process (Katz, 1970), or contraction-related metabolic events in the cell (Danforth, 1964; Nocker, 1964; Edgerton et al., 1975).

Oxidative metabolism is ideally suited to meet the energy requirements of muscular work since 18 fold more energy can be derived from glucose if oxygen is the final hydrogen acceptor than when pyruvate serves as the substrate (Lehninger, 1970). In addition, the overall plan of oxidative metabolism has as its basis the Kreb's citric acid cycle (CAC) and oxidative-phosphorylation, the final common pathways in which all fuel molecules of the muscle cell, carbohydrates, fatty acids, and amino acids are ultimately degraded to water (H_2O) and carbon dioxide (CO_2) (Lehninger, 1970).

The oxidative demands on muscle are known to increase by thirty fold or more in the transition from rest to maximum contractile activity (Chapler and Stainsby, 1968; Stainsby and Fales, 1973; Hirche et al.,

1971, 1975). This energy demand for the predominantly oxidative resynthesis of adenosine triphosphate (ATP) is associated with 2 realignments in the CAC, namely, increased flux through the CAC enzyme complexes (Keul et al., 1972) and a sustained increase in the concentrations of the CAC intermediate pool (Keul et al., 1972; Edington et al., 1973; Koziol and Edington, 1975).

Such realignments in oxidative metabolism necessitate precise interaction of glycolysis, the malate-aspartate shuttle and the citric acid cycle (Figure 1). The malate-aspartate shuttle appears to be a principle way in which glycolytically-generated reducing equivalents are transported from cytosol to mitochondria in skeletal muscle (Koziol and Edington, 1975). Hence, intramitochondrial α-ketoglutarate (αKG) and oxaloacetate (OAA) are at metabolic crossroads and the metabolic fates of these metabolites are determined not only by reactions in the CAC, but also by cytosolic events which share these key metabolites. The CAC, therefore, appears to be regulated such that entry of OAA and αKG into the 1^{st} and 2^{nd} spans of the CAC, respectively, or diversion into the malate-aspartate shuttle may occur (Figure 1).

Net gain in the total tissue CAC intermediate pool cannot be a direct result of increased rate of acetyl-CoA entry into the cycle since two molecules of CO_2 are formed per cycle turnover and net additional OAA is not generated (Keul et al., 1972). Increased tissue levels of the CAC intermediate pool therefore requires precursors which are not originally part of the CAC pool. A number of CAC anaplerotic ("filling up") mechanisms for skeletal muscle have been proposed with this in mind.

Fixation of CO_2 as a mechanism for converting mono- to dicarboxylic acids has a low activity in skeletal muscle because of the absence of

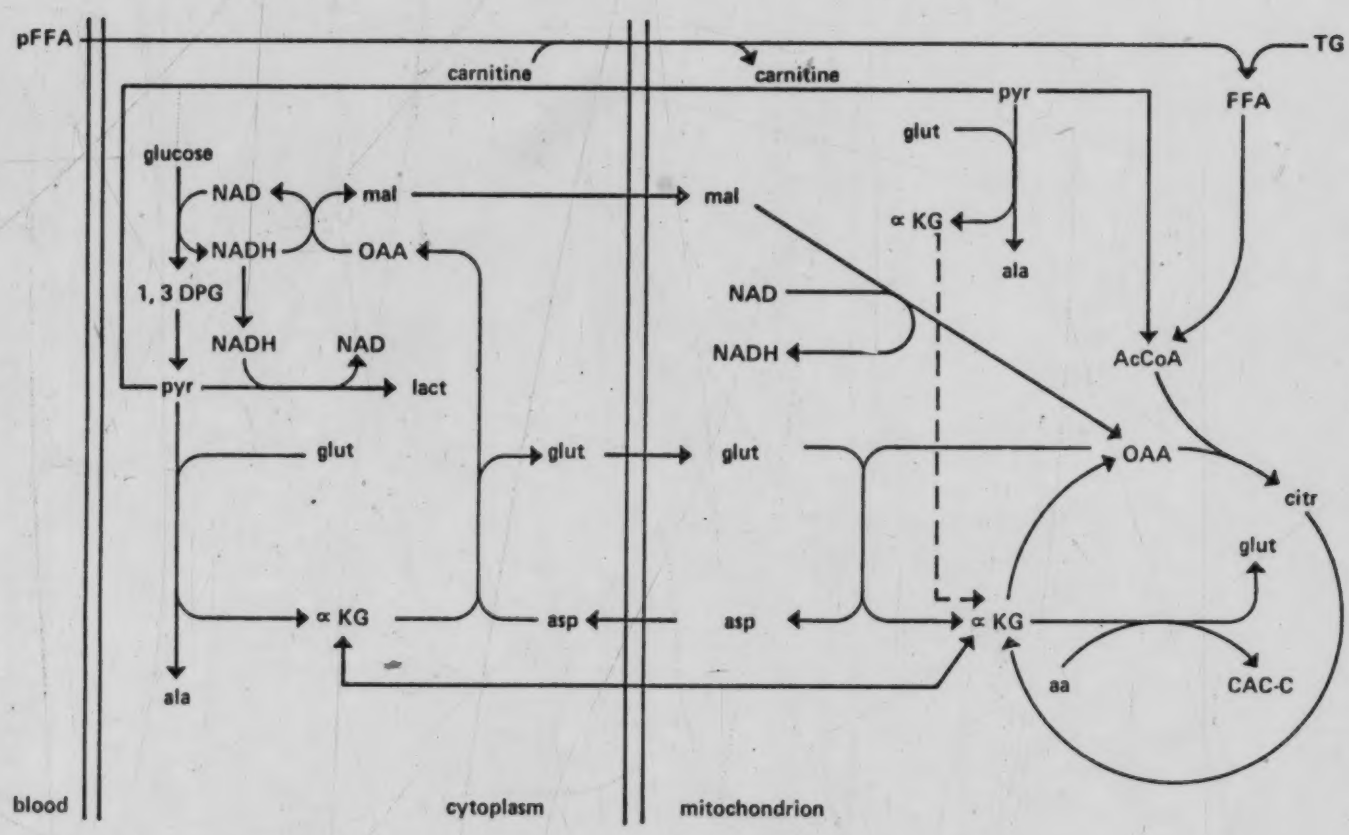


Figure 1 Metabolic flux scheme showing the relationship between glycolysis, the malate-aspartate shuttle, ß-oxidation, amino acid oxidation and the citric acid cycle in skeletal muscle (refer to the abbreviation key on the next page)

Abbreviation key for Figure 1 :

pFFA	plasma free fatty acids
FFA	free fatty acids
TG	triglycerides
AcCoA	acetyl CoA
OAA	oxaloacetate
citr	citrate
αKG	α-ketoglutarate
mal	malate
aa	amino acid
asp	aspartate
glut	glutamate
ala	alanine
1,3DPG	1,3 diphosphoglycerate
pyr	pyruvate
lact	lactate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
CAC-C	citric acid cycle carbon skeleton

pyruvate carboxylase (Equation 1: Lehninger, 1970) and the limited activity of malic enzyme in the direction of malate synthesis (Equation 2: Molé et al., 1973).

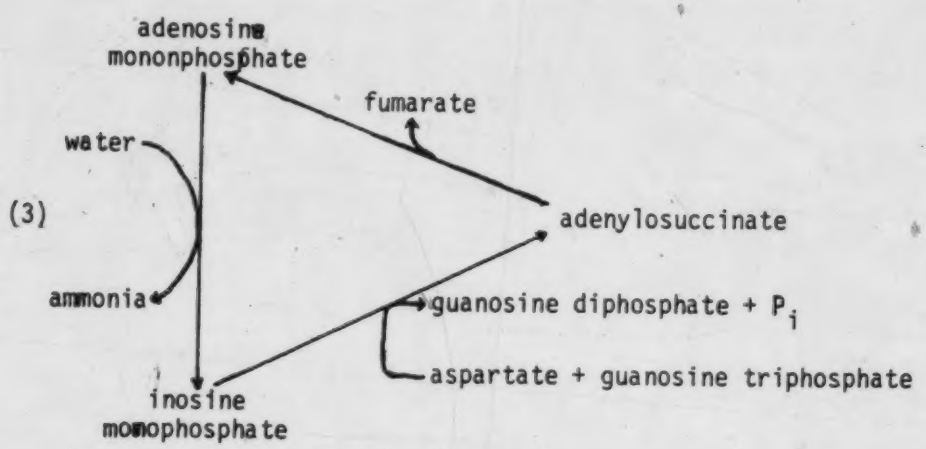
(1) Pyruvate + CO_2 + ATP $\rightleftharpoons$ oxaloacetate + ADP + P_i

(abbreviation key: ADP, adenosine diphosphate; P_i, orthophosphate)

(2) Pyruvate + CO_2 + NADPH + H^+ $\rightleftharpoons$ Malate + NADP

(abbreviation key: NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, oxidized nicotinamide adenine dinucleotide phosphate).

The "purine nucleotide cycle" may play a role in net generation of CAC intermediates (Equation 3: Lowenstein, 1969; Lowenstein and Tornheim, 1971).



Skeletal muscle has been reported to generate ammonia during contractile activity (Lowenstein, 1969), however, the quantitative significance of this pathway to the total CAC pool remains obscure, particularly because excessive ammonia production by muscle may have toxic effects (Carlsten

et al., 1967).

It has been generally accepted that primary augmentation of the skeletal muscle CAC intermediate pool occurs via transamination and subsequent catabolism of the large pool of free amino acids in the muscle (Bartley et al., 1968; Keul et al., 1972; Newsholme and Start, 1973). In particular, increases in the levels of OAA, malate and αKG, associated with a fall in the level of glutamate and a rise in the level of aspartate, have been noted (Borst, 1962; Lefèbvre et al., 1972; Edington et al., 1973; Koziol and Edington, 1975). In addition to these events, it has been established that glutamate will transaminate with pyruvate to form αKG and alanine in skeletal muscle (Equation 4: Carlsten et al., 1962; Young, 1970; Hider and Meade, 1972; Aikawa et al., 1973; Edington et al., 1973; Felig, 1973; Molé et al., 1973; Ellis et al., 1974; Grubb and Snarr, 1974, Odessey et al., 1974).

(4) Pyruvate + glutamate $\rightleftharpoons$ αKG + alanine

Aikawa et al. (1973) and Odessey et al. (1974) have shown that de novo alanine from glutamate-pyruvate transamination represents the quantitatively important end product of amino acid metabolism in skeletal muscle. Odessey and coworkers (1974) have extended this to the conclusion that alanine production by skeletal muscle in vitro accounted for all amino nitrogen from the transamination and subsequent oxidation of the branched chain amino acids, leucine, isoleucine and valine.

It is clear from an inspection of the carbon stoichiometry that a simple transamination of any amino acid and subsequent metabolism cannot give rise to a net increase of the CAC intermediate pool nor can alanine be a direct product of single transaminations involving the branched chain amino acids. This dilemma may be resolved by

consideration of the work of Davis et al. (1972) and Safer and Williamson (1973) with isolated, perfused rat hearts. Coupled transamination (Equations 5, 6 and 7: Safer and Williamson, 1973) by glutamate-oxaloacetate transaminase (GOT) of the malate-aspartate shuttle and glutamate-pyruvate transaminase (GPT) is capable of forming net CAC intermediates in the cytosolic compartment from two metabolites which are not directly part of the CAC pool (Figure 1).

(5) aspartate + αKG $\rightleftharpoons$ OAA + glutamate

(6) glutamate + pyruvate $\rightleftharpoons$ alanine + αKG

(7) net: aspartate + pyruvate $\rightleftharpoons$ alanine + OAA

Moreover, the free amino acids which transaminate with αKG as a preliminary step to oxidation (Bartley et al., 1968), may also couple with GPT (Davis et al., 1972) and through ancillary reactions (Equations 8, 9 and 10) yield a net effect of the following type:

(8) free amino acid + αKG $\rightleftharpoons$ CAC - carbon skeleton + glutamate

(9) glutamate + pyruvate $\rightleftharpoons$ alanine + αKG

(10) net: free amino acid + pyruvate $\rightleftharpoons$ CAC - carbon skeleton + alanine

The alternative pathways of pyruvate metabolism during aerobic exercise in skeletal muscle are supportive of the concept of coupled transaminations in skeletal muscle. Glycogenolysis to pyruvate in working skeletal muscle occurs at a rate dependent on the relative work intensity (Saltin and Karlsson, 1971a, b; Hultman and Bergstrom, 1973; Saltin, 1973; Gollnick et al., 1974a), yet high rates of acetyl-CoA production from β-oxidation in submaximal exercise inhibits pyruvate oxidation through the CAC (Garland et al., 1969; Newsholme and Start, 1973). It has been

proposed that alanine production from pyruvate in human skeletal muscle at rest may occur at 35-60% of the rate of lactate production (Felig and Wahren, 1971a, b). This relationship is not maintained in submaximal exercise where lactate production by muscle has been shown to rise transiently during the initial stages of contraction and thereafter decreases in both in vivo human muscle (Costill, 1970, Ahlborg et al., 1974) and in situ mammalian muscle (Chapler and Stainsby, 1968; DiPrampero et al., 1969; Hirche et al., 1971, 1973). Alanine production on the other hand, undergoes a sustained increase during submaximal aerobic exercise, the magnitude of which appears to be related to the work intensity (Felig and Wahren, 1971a, b, 1974; Ahlborg et al., 1974). Although incomplete, the above information does suggest that excess pyruvate is created beyond the capacity or need of the muscle to oxidize this substrate.

The functional significance of alanine production from pyruvate has previously been associated primarily with the "glucose-alanine cycle" (Felig and Wahren, 1971; Felig, 1973), an analog of the Cori cycle in which peripherally derived alanine provided a gluconeogenic precursor to the hepatic tissues. Also, alanine was considered of importance for non-toxic transport of ammonium (i.e., amino groups) from muscle to liver for urea production (Carlsten et al., 1967).

In light of the evidence supporting coupled transamination reactions in skeletal muscle, alanine production may have significance for increased flux in the CAC as well as the repletion and maintenance of the elevated concentrations of the total CAC intermediate pool which are associated with exercise (Keul, et al., 1972, Molé et al., 1973). Furthermore, alanine production may also act via coupled transaminations as an indirect alternative pathway to lactate production in the reoxidation of

reduced pyridine nucleotides (Figure 1) (Felig and Wahren, 1971a; Safer and Williamson, 1973).

RATIONALE FOR THE USE OF AN ANIMAL MODEL. Biochemistry of exercise has passed from the state of a descriptive science to the stage where quantitative problems are increasingly important. The fundamental questions of current interest in muscle metabolism concern those factors which may limit the metabolic sequence in muscular activity (Keul et al., 1972; Howald and Poortmans, 1975). These limiting metabolic factors may include the following: 1) the amount of substrate transported to the muscle cell, 2) the amount of substrate in the muscle, itself, 3) the amount of metabolites produced and removed from the cell, 4) substrate turnover in the cell, i.e., the rates at which substrates are transformed by the enzyme complexes, 5) the nature of the control mechanisms which adjust substrate turnover rate in the cell and 6) the contractile process.

Although it is very easy to measure the limitations of man's capacity for exercise, only a portion of the above-mentioned factors may be observed in the musculature of human subjects due to technical limitations in human research. The surgical implantation of catheters into the vasculature of human limbs for the measurement of arterial-venous differences of metabolites has provided substantial information as to muscle metabolism. However, the limitations of this method include interpretive problems as a result of extraneously involved tissues such as skin and adipose tissue in the limb. Also, differentiation of one muscle from the total muscle mass of the limb is impossible. A practical technique for obtaining biopsy specimens from muscle has also advanced our knowledge of muscular events in exercising man. Nonetheless, this technique has inherent limitations in the human model. The time factor associated with obtaining biopsy material and the small size of these samples limits

the technique to quantitation of fewer, less labile metabolites.

The whole area of human research is further complicated by the ethics of surgical intervention and the problems of volitional exercise responses. Metabolic occurrences in muscle have therefore been examined extensively in electrically stimulated *in situ* mammalian muscle preparations (Omachi and Lifson, 1956; Kugelberg and Edström, 1968; Piiper *et al.*, 1968; Corsi *et al.*, 1969; Edgerton *et al.*, 1970; Hirche *et al.*, 1970a, b; Stainsby, 1970; Baldwin and Tipton, 1972; Koziol and Edington, 1975; Morganroth *et al.*, 1975; Horstman *et al.*, 1976). Yet, animal experiments should be referred to, only in-so-far as they clarify the transfer of energy during muscular activity in man.

RATIONALE BEHIND THE PRESENT STUDY. No systematic approach has been used in quantitating alanine production from isolated, fatigue resistant (oxidative) muscle under various respiratory demands, nor has the production of alanine under these conditions been related to the flux and repletion of the CAC in skeletal muscle.

The dog gastrocnemius muscle consists entirely of fatigue resistant (oxidative) muscle fiber types (Maxwell *et al.*, 1975). The direct maximal electrical stimulation of this muscle will result in the recruitment of all muscle fibers in the muscle (Kugelberg and Edström, 1968). Evidence indicates that variations in frequency of electrical stimulation (Chapler and Stainsby, 1968) and variations in the intratrain stimulation rate (DiPrampero *et al.*, 1969; Horstman *et al.*, 1976) are effective in producing a continuum of graded metabolic responses up to the maximal oxidative capacity of a muscle. For these reasons, varied electrical input to isolated, *in situ* canine gastrocnemius muscle preparations was employed as the experimental model for quantitative study of the relation-

ship between alanine production and the metabolic realignments occurring in the CAC during two levels of oxidative contractile activity.

METHODOLOGY

ANIMAL CARE. Adult mongrel dogs of both sexes, 15-30 kg bodyweight, were acquired through the Health Sciences Animal Center (University of Alberta) as unclaimed dogs from Edmonton municipal dog pounds under the authority of Alberta provincial legislation. Each dog was quarantined for a 2 week revitalization period at the University farm during which time they were bathed, de-fleaed, wormed and vaccinated against canine distemper and hepatitis. Their diet consisted of Burger Bits (Standard Brands, Montreal) (Appendix II, Table 1) and water ad libidum. Animals were moved to pre-operative quarters (Surgical Medical Research Institute) 24 hours prior to experimentation, given water ad libidum and fasted overnight. Dogs were weighed immediately prior to experimentation.

ANESTHESIA. Anesthesia was induced with a mixture of room air and halothane (5%) (Fluothane, Ayerst, McKenna and Harrison Ltd., Montreal) delivered to a muzzle cone. Immediately following induction, a modified endotracheal tube was inserted and linked to the anesthetic gas mixture which was adjusted to maintain the plane of anesthesia (1.5-2% halothane in air). Preliminary study of halothane anesthetized dogs had revealed subnormal values for PO_2 in arterial blood (35-60 mmHg). Therefore, a 3 mm polyethylene tube was fixed to the exterior surface of the endotracheal tube such that a continual flow of oxygen:carbon dioxide (95:5) was also administered to the animal. The flow of oxygen:carbon dioxide was adjusted to maintain a normal physiological range of oxygen partial pressure in arterial blood (P_AO_2:80-110 mmHg) based on a previously established linear relationship between gas flow and bodyweight of the

animal.

SURGERY. The gastrocnemius muscles of the right and left hind-limbs were exposed through medial incisions extending in a straight line from the frontal aspect of the thigh, along the longitudinal axis of the gastrocnemius muscle to the ankle. Minor bleeding was controlled by electrical cautery (Birtcher Electro-surgical Unit, Model 755). Major subcutaneous vasculature was doubly ligated and cut between ligatures. Skin, connective tissue and fat covering the muscles were then separated and retracted. The inserting tendon of the semitendinosus muscle was doubly ligated and severed between ties. The gracilis and semimembranosus muscles were isolated for 5-8 cm of their length from their points of insertion, doubly ligated and cut between ligatures; a 5-8 cm length of the underlying femoral venous blood flow from the gastrocnemius muscle was isolated by ligation of all venous vasculature not associated with blood flow of the muscle. The patency of this technique in isolating venous return was demonstrated by arteriography in preliminary study (Appendix III, Figure 3). A 2-3 cm segment of the sciatic nerve was isolated by blunt dissection near the muscle, doubly ligated and cut between ties.

TENSION MONITORING. The distal portion of the left common calcanean tendon and the calcaneus were exposed through an incision running posteriorly 5 cm along the line of the tendon to 5 cm distal to the tuber calcanei. Bleeding was controlled by cautery and/or ligation. A $^{1}/_{8}$ X 3 inch drill bit was passed perpendicularly through the medial aspect of the calcaneus and the calcaneus was divided from the foot at a point just distal to the drill site. The calcaneus and 2-3 cm of the tendon were blunt dissected free of surrounding tissues (Appendix III, Figure 6). A $^{1}/_{4}$ X 10.5

inch drill bit was passed perpendicularly through the femur at the mid-point of the origin of the gastrocnemius muscle (Appendix III, Figure 2).

The angles at the left hip and knee were adjusted to approximate a right angle to ensure uninhibited blood flow (Hirche et al., 1970a) and the femur was spatially fixed by rigidly clamping the drill bit to a platform under the dog's leg on which the tension monitor was mounted (Appendix III, Figure 7). The calcaneus was connected via a cable to a tension-calibrated leaf-spring linked in series to a displacement transducer (Appendix III, Figures 6, 8). The effects of the mass of the lower limb and foot on the tension monitor were neutralized by suspending the foot from an overhead point (Appendix III, Figure 4). Resting tension of the muscle was adjusted to 1.2 Kg since preliminary study revealed maximum tension development for an applied stimulus was obtained in this range.

Tension records were produced continually on a Beckman 4 channel type R Dynograph (Beckman Instruments, Inc., Offner Division, Schiller Park, Illinois) (Figure 2 and Appendix IV, Figure 1). Response in this monitoring system was established as being linear (±2%) up to tensions of 20 Kg during tension calibration experiments (a preweighed bucket was suspended via a pulley and cable, and water of known temperature and volume was added).

BLOOD FLOW. A superficial branch of the right femoral artery was surgically isolated on the medial aspect of the thigh at a point where the branch was palpable in crossing the pectineus muscle. This artery was cannulated with 1.5 mm I.D. polyethylene tubing such that the end of the cannula was adjacent to, but not interrupting femoral arterial blood flow. Arterial blood samples were collected from this source via a 3 way Luer-Lok valve (Figure 2).

The cephalic vein of the left fore-limb was surgically isolated,

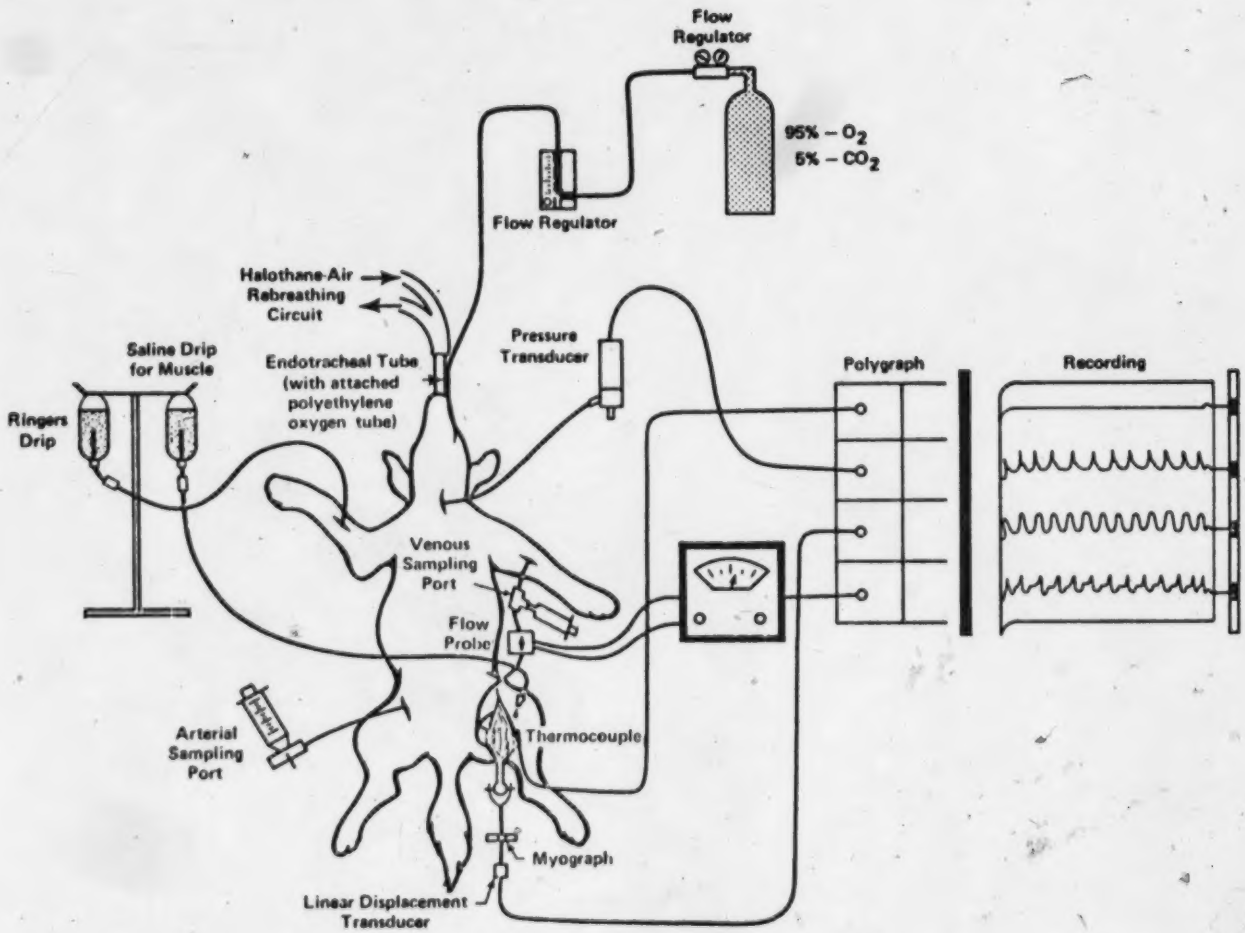


Figure 2 Schematic representation of the isolated, in situ dog gastrocnemius muscle preparation

tied distally and cannulated with 3 mm I.D. polyethylene tubing pre-filled with normal saline. The left femoral vein was tied proximally and cannulated with identical tubing. Occlusion time for blood flow was 30-60 sec during the latter procedure. Venous flow from the muscle was passed through a 3 mm external flow-through probe (electro-magnetic, square-wave flowmeter, Model SWF-1M, Zepeda Instruments, Seattle) and returned to the cephalic vein of the left fore-limb (Appendix III, Figure 7). A slight downhill gradient was maintained in the femoral-cephalic direction (Appendix III, Figure 7). A 3-way Luer-Lok valve between the flow probe and the catheter of the cephalic vein was used to flush trapped air from the system prior to establishing flow. This same 3-way valve was employed in collecting venous blood samples from the left gastrocnemius muscle (Appendix III, Figure 7).

Blood flow was recorded continually on the Beckman 4 channel recorder (Figure 2 and Appendix IV, Figure 1). Preliminary study of this flow monitoring system indicated a linear response up to flows of 200 ml/min. Blood flow was calibrated daily employing the collection of a timed volume of venous blood from the 3-way valve in the system, while zero drift was accounted for with brief venous occlusion prior to and following the experiments.

ANTICOAGULANT ADMINISTRATION. Coagulation of blood in all cannulae was inhibited by the administration of sodium heparin (1000 U/ml of saline) (Sigma Chemical Co., St Louis, Mo.) (initial dosage (ml) = 0.5 times the dog's body weight. Booster doses (= initial dosage times 0.4) were administered hourly thereafter.

Note: All surgery with the exception of cannulations was completed prior to heparin administration to prevent excessive blood loss during surgical procedures.

BLOOD PRESSURE. The cephalic vein of the right fore-limb was surgically isolated, tied distally and cannulated with a saline filled 3 mm polyethylene catheter. A gravity aided Ringer's I.V. drip was introduced to this cannula for the duration of the experiment (1 drop/sec; 4 ml/min) in order to offset hemoconcentration associated with experiments of this nature and duration and also to offset fluid and electrolyte loss associated with blood sampling.

The left common carotid artery was surgically isolated, tied distally and catheterized with 3 mm polyethylene tubing which was initially coupled to a Stathan Pressure Guage (Stathan, Hato Ray, Puerto Rico) and flushed with saline to remove all traces of air. Blood pressure was monitored continually at this site via the Beckman 4 channel recorder (Figure 2 and Appendix IV, Figure 1). The Stathan pressure transducer was calibrated regularly with a manometer (Thistle, Model C80216).

TEMPERATURE MAINTENANCE. Rectal temperature was recorded from a mercury thermometer immediately following induction of anesthesia. A low temperature heating pad was placed over the animal's thorax and upper abdomen and adjusted to maintain the initial recording of body temperature.

Following surgical isolation of the right gastrocnemius muscle, a saline moistened guaze was placed over the exposed muscle surface. The incision was closed temporarily with clamps and spontaneous rewarming of the muscle to body temperature occurred. Temperature of the exposed left muscle was monitored with a mercury thermometer inserted into the space enclosed by the tibia and gastrocnemius muscle belly. An incandescent heat lamp was employed in regulating muscle temperature in accordance with observed rectal temperature. The exposed surface of the muscle was continually flushed with a slow isotonic saline drip. Excessive accumulation of fluid in the cavity surrounding the muscle was

avoided by placing a 10 guage needle-drain through the skin forming the base of the cavity (Appendix III, Figure 5).

EXPERIMENTAL PROTOCOL. Following completion of the details of the surgical preparation, a post-operative recovery period of at least 30 min was observed. During this time the animal was stabilized as to resting blood flow, acceptable rectal and muscle temperatures, blood pressure and femoral P_AO_2. Further, the animal was then randomly assigned to one of four groups among which the intensity and duration of stimulation were varied. The design of individual experiments is outlined in FIgure 3.

Groups were designated as to severity of exercise (mild (M) or severe (S)) and as to duration of the experimental period (20 or 65 min). Hence "M65" indicated a group of dogs which received mild electrical stimulation to the left gastrocnemius muscle for a period of 65 minutes. In all experiments, pre-exercise tissue sampling trauma to the left muscle was injudicious, therefore the right gastrocnemius provided an non-stimulated contralateral control in which the blood supply and nerve were surgically altered as in the left experimental leg (Figure 3).

STIMULATION. The isolated left gastrocnemius muscle was stimulated directly using two 1 cm stainless steel needle electrodes placed 2 cm apart in the proximal $^1/_3$ of the medial head of the muscle (Appendix III, Figure 5). The applied stimulus was in the form of D.C. square waves, 5 m sec in duration at a frequency of 22/sec and at maximal voltages (Electronic Stimulator, Model 751, Amercan Electronic Labs, Inc. Philadelphia, PA: modified to deliver trains). Two rhythmic tetanic contraction forms were used; trains of impulses lasting 0.08 sec "on" and 2.7 sec "off" were designated as a mild stimulus (M) while trains of impulses lasting 1.4 sec "on" and 2.7 sec "off" were considered as a

Group Designation	Impulse Trains		TIME (min)					
			Rest	15	20	40	60	65
M20 (n = 6)	0.08 sec "on" 2.7 sec "off"	stimulated left muscle	B*	B*	FC/WM			
		resting right muscle			FC/WM			
M65 (n = 6)	0.08 sec "on" 2.7 sec "off"	stimulated left muscle	B*	B	B	B	B*	FC/WM
		resting right muscle						FC/WM
S20 (n = 6)	1.4 sec "on" 2.7 sec "off"	stimulated left muscle	B*	B*	FC/WM			
		resting right muscle			FC/WM			
S65 (n = 6)	1.4 sec "on" 2.7 sec "off"	stimulated left muscle	B*	B	B	B	B*	FC/WM
		resting right muscle						FC/WM

Figure 3 Experimental protocol demonstrating intensity-duration combinations and sample intervals; (B*)28 ml blood samples :(B)8 ml blood samples :(FC/WM)freeze-clamp muscle sample followed immediately by whole muscle extraction

severe stimulus (S). Preliminary study revealed that stimulus S elicited maximal blood flow and oxygen uptake for the muscle preparation under present experimental conditions, while stimulus M elicited an oxygen uptake approximately 50% less than that observed with stimulus S.

BLOOD SAMPLES AND ANALYSES. Simultaneous paired blood samples were drawn anaerobically at specified intervals (Figure 3) via the right femoral arterial catheter and from the isolated left venous flow circuit (Figure 2) into lubricated glass syringes. Stasis in drawing samples was avoided by controlling collection rate in line with observed flow rate. At blood sample intervals indicated by B* in Figure 3, paired blood samples of 28 ml each were collected; all other paired blood samples were of 8 ml each. The additional 20 ml at intervals B* were required for amino acid analysis. In dogs which were studied for 65 minutes, this protocol required the collection of a total of 136 ml of blood over the experimental period. Archer (1965) has indicated that 9 ml/Kg is the safe limit for acute blood loss in a dog without the occurrence of notable physiological changes. Preliminary study in the present investigations revealed minimal changes in arterial hemoglobin, hematocrit and plasma proteins under the practiced regimen.

Immediately following collection, blood samples were analyzed for oxygen partial pressure (PO_2) and pH (Radiometer blood gas apparatus - pH meter 27, Radiometer Co., Copenhagen). Assessments of hematocrit (Hct) (microhematocrit technique) and hemoglobin (Hb) (cyanmethemoglobin technique) were also conducted. Remaining blood samples were then centrifuged at 2000 rpm for 15 min and the plasma was partitioned and frozen pending subsequent analyses.

Glucose (Sigma Kit 510), pyruvate (Sigma Kit 726) and lactate (Sigma Kit 826) were determined enzymatically while plasma free fatty acids were

analyzed according to Pinelli (1973). Plasma proteins were assayed colourimetrically by the Biuret reaction. Glutamate, aspartate, alanine and lysine in plasma were determined by the automated ion-exchange chromatographic technique after deproteinization of plasma with 1% picric acid (Beckman Instruction Manual, Model 121 Automatic Amino Acid Analyzer, 1969). A minor modification of the above technique for sample preparation was employed in that the effluent from the Dowex 2 - x8 (200 mesh) resin bed was concentrated by freeze-drying rather than by rotary evaporation. This was a viable alternative and necessitated by unavailability of a rotary evaporator.

TISSUE SAMPLE AND ANALYSES. At the termination of each experiment, freeze clamp tongs (Appendix III, Figure 9), precooled to the temperature of liquid nitrogen, were employed to rapidly freeze a 2-3 gm sample in the center of the medial head of the left and right gastrocnemius muscles. These samples were then rapidly excised by trimming about the tong faces, immersed briefly in liquid nitrogen, placed in polyvinyl containers (precooled in dry ice) and stored in a deep freeze (-60°C) for later analysis. In preliminary investigation, a copper-constantan thermocouple was inserted into the center of the proposed sample site. Stainless steel tongs precooled in liquid nitrogen could be applied to the muscle at the moment of cessation of a tetanus such that muscle temperature declined to -20°C within 1 sec and -50°C before 2 sec.

The left and right gastrocnemius muscles were quickly isolated by severing the common calcanean tendons and working in the distal-proximal direction until only the blood supply of the muscles remained intact. This was clamped, severed and the muscles removed to isotonic saline where visible fat, connective tissue and unrelated muscle tissues were removed. The muscle was divided into its three component heads (medial,

lateral and flexor) by application of pressure along the lines of least resistance. Further visible fat and connective tissue were removed. The muscle heads were individually separated from the calcanean tendon at the muscle-tendon interface and the muscle tissues of the individual heads were weighed.

Each head of both muscles was bisected transversely at its mid-point and longitudinal core samples of sufficient size were dissected free. These samples were immersed in hexane, precooled with liquid nitrogen, and placed in capped polyvinyl containers precooled in dry ice. These samples were stored at -60°C for subsequent analysis.

Freeze-clamped muscle samples were weighed in a cold chamber with care taken to preserve refrigeration, i.e., the entire sample was briefly immersed in liquid nitrogen, rapidly weighed and re-immersed in liquid nitrogen. Samples were then mechanically fragmented in liquid nitrogen into samples of sufficient size for free amino acid (0.8-1.2 gm) and muscle triglyceride (60-120 mg) analysis, and stored at -60°C.

Free amino acids (glutamate, aspartate, alanine, lysine) were determined by automated ion-exchange chromatography after employing the following modification of the sample preparatory techniques as outlined in the Beckman Instruction Manual, Model 121 Automatic Amino Acid Analyzer (1969). After weighing, the tissue was immersed in liquid nitrogen and mechanically ground to a fine powder. The powdered material was transfered with a precooled spatula to 10 ml of 1% picric acid in a centrifuge tube which was then capped and mechanically shaken for 2 min. As with the plasma amino acid analysis, effluents from the Dowex resin bed were concentrated by a freeze-drying procedure.

Determination of muscle triglycerides required slight modification of the methods outlined by Carlson (1963) and Carlson et al. (1971).

Appropriate quantities of sample were quickly weighed, and ground in liquid nitrogen employing a mortar and pestle. The pulverized material was transferred to 2 ml of methanol for extraction of muscle lipids, shaken for 2 min and 4 ml of chloroform and 6 ml of saline were added. After overnight equilibration of the phases, the chloroform phase was removed and treated according to Carlson et al. (1971).

The frozen muscle core samples were divided for glycogen and histochemical analyses. Glycogen was isolated from muscle tissue and analyzed by the method of Lo et al. (1970). Tissue blocks for histochemical staining were mounted on chucks in a cryostat at -25 to -30°C. Serial sections of 10μ for myofibrillar adenosine triphosphatase (myosin ATPase) and reduced nicotinamide adenine dinucleotide diaphorase (NADH diaphorase) and 16μ for periodic acid-Schiff (PAS) staining were cut and mounted on microscope slides. These sections were air dried for 24-48 hours prior to appropriate staining (Dubowitz and Brooke, 1973). The procedure followed in staining for myosin ATPase was modified to preincubate the sections at pH 10.4. Improved definition of capillarization and black and white differentiation was obtained by this procedure.

PREPARATION VIABILITY. Individual experiments were assessed for vital changes and loss of viability as a result of surgical trauma, anesthesia, experiment duration, blood loss (sampling trauma) and/or temperature changes. The bases for these assessments included preliminary inspection of the following parameters: blood flow P_AO_2, arterial pH, Hb, Hct, plasma proteins, sample hemolysis, substrate/metabolite arterial concentrations, carotid arterial blood pressure (systolic and diastolic) and rectal and muscle compartment temperatures. Abnormal absolute values or large changes between resting and the ultimate sample intervals

were evaluated. While single unusual values did not result in deletion of an experiment from the statistical analyses, combinations of factors were guaged subjectively as contra-indicators. Two dogs were rejected based on these assessments (refer to pp 37, Results).

CALCULATIONS

Oxygen Consumption. The PO_2-Oxygen Saturation % Nomogram (Appendix V, Figure 1) was employed in determining % O_2 saturation of arterial and venous blood from PO_2 and pH according to Astrup (1965). Oxygen consumption ($\dot{V}O_2$) was then calculated employing the Fick principle (Davidsohn and Henry, 1969).

$$\dot{V}O_2(ml/min) = \frac{Hb \times 1.36}{100} (HbO_2A - HbO_2V) \cdot \text{flow rate}$$

where:

$\frac{Hb \times 1.36}{100}$ is the O_2 capacity (ml/min) of blood; Hb is in g %

$(HbO_2A - HbO_2V)$ is the arterial-venous difference for the oxygen content of blood (ml/min); oxygen content of blood = % O_2 saturation X O_2 capacity

flow rate is the venous blood flow rate in ml/min.

Arterial-Venous Differences. Net uptake or output of blood-borne materials across the muscle was determined by the Fick principle (Schlein et al., 1973).

$$\text{net uptake or output of S} = ([S]_A \cdot F_A - [S]_V \cdot F_V)$$

where:

$[S]_A$ is the concentration of substance S in arterial blood

$[S]_V$ is the concentration of substance S in venous blood

F_A is the arterial blood flow rate (ml/min)

F_V is the venous blood flow rate (ml/min).

Flow rate was corrected for rate of water loss from plasma during transit through the muscle according to Schlein et al. (1973).

$$\text{Arterial flow rate} = \text{venous flow rate} \times \frac{TP_V}{TP_A}$$

where:

TP_V is the total protein concentration in venous plasma

TP_A is the total protein concentration in arterial plasma.

Uptake or output data for glucose, pyruvate, lactate, alanine, glutamate, lysine and aspartate were calculated from venous and arterial blood flow rates. Free fatty acid arterial-venous differences were calculated from venous and arterial plasma flow rates where:

$$\text{venous plasma flow} = \text{venous blood flow} \times (1 - \text{Hct})$$

$$\text{arterial plasma flow} = \text{venous plasma flow} \times \frac{TP_V}{TP_A}$$

Estimation of de Novo Synthesis of Alanine from Glutamate-Pyruvate Transamination. The de novo production of alanine from glutamate-pyruvate transamination in muscle was estimated as follows (modified from Felig and Wahren, 1971a):

μM of alanine/g/min which are produced by glutamate-pyruvate transamination

$$= [\text{ala A-Vd}_t \pm \frac{(\frac{lys_t}{lys_R} \cdot ala_R) - ala_t}{t} - \text{lys A-Vd}_t]$$

where:

ala A-Vd_t is the alanine output from muscle (μM/g/min) at time (t) = 15 or 60 min

lys A-Vd_t is the lysine output from muscle (μM/g/min) at time (t) = 15 or 60 min

lys_t is the free lysine content of muscle (μM/g) at time (t) = 20 or 65 min

lys_R is the free lysine content of muscle (μM/g) under resting conditions

ala_t is the free alanine content of muscle (μM/g) at time (t) = 20 or 65 min

ala_R is the free alanine content of muscle (μM/g) under resting conditions

t is the time interval (20 or 65 min) appropriate to the time at which the arterial-venous differences of alanine and lysine were observed (15 or 60 min sample intervals, respectively)

$|\ |$ denotes the absolute quantity of the enclosed expression

± notation indicates appropriate correction for over- or under-estimation of alanine output from muscle relative to lysine output from muscle (see example following).

The theory supporting estimation of the alanine production from glutamate-pyruvate transamination in muscle is as follows: lysine and alanine residues occur in equal proportions in structural and soluble proteins of rabbit skeletal muscle (i.e., 1954; Lowey and Cohen, 1962; Koizumi, (1974). Gross interspecies differences between rabbit and dog muscle proteins should not be evident since functional integrity of protein is highly correlated with primary structure (i.e., amino acid sequence) (Lehninger, 1970). Turnover of alanine and lysine residues occur at similar

rates in resting rabbit skeletal muscle proteins (Koizumi, 1974) (i.e., alanine and lysine residues are equally available to the protein catabolic mechanisms of skeletal muscle). Lysine is not catabolized or synthesized in skeletal muscle (Bartley et al., 1968; Odessey et al., 1974). Therefore, the extent to which alanine output from muscle exceeds lysine release (ala A-Vd - lys A-Vd) was an initial index of alanine production from sources other than the proteolytic processes (i.e., glutamate-pyruvate transamination).

Muscle tissue contents of alanine and lysine at rest are proportional to their respective arterial concentrations (Table 7 and Appendix VII, Table 2), suggesting that loss of these amino acids from tissue concentrations occur at similar relative rates in resting muscle. Arterial concentrations of alanine and lysine demonstrated parallel changes with exercise (Appendix VII, Table 2). Therefore, it was assumed that muscle contents of these amino acids should change in parallel during exercise as evidenced by comparisons of left to right ratios (Table 7). However, non-parallel changes in muscle concentrations of these amino acids occurred. This difference was attributed to different rates of alanine production from glutamate-pyruvate transamination since proteolytic input to the free pools of alanine and lysine was assumed to be equal. This imbalance between input and output and its subsequent effect on alanine "escape" from muscle was estimated from temporal changes in muscle tissue contents of lysine, i.e., $(\frac{lys_t}{lys_R} \cdot ala_R)$ estimates the tissue content of alanine at time (t) that should have been apparent if parallel tissue concentration changes had occurred for lysine and alanine. Subtraction of the observed ala_t from the estimated ala_t and division by time (t) estimates the absolute differential rate of loss of alanine occurring per t minutes of exercise. The alanine arterial-venous difference was cor-

rected for this differential by addition or subtraction of the appropriate absolute differential rate, i.e., where lysine content of muscle was diminished relatively more than the alanine content of muscle, as evidenced by left to right ratios, addition was indicated. Subtraction was indicated by an opposite effect.

Estimation of the Percentage of Muscle Oxygen Uptake Accounted for by Carbohydrate Oxidation. The percentage of the oxygen uptake of the muscle accounted for by total carbohydrate oxidation was calculated as follows (modified from Chapler and Stainsby, 1968):

$$\%\dot{V}O_2(CHO) = \frac{[gluc + glyc - (\frac{L}{2} + \frac{P}{2} + \frac{A}{2})] \cdot 6}{\dot{V}O_2} \cdot 100$$

where:

gluc is the glucose uptake of the muscle (mM/g/min)

glyc is the rate of glycogen catabolism expressed as glucosyl units (mM/g/min)

$\frac{L}{2}$ is the lactate output from muscle (mM/g/min) divided by 2 since 1 mole of glucose may produce 2 moles of lactate, pyruvate or alanine

$\frac{P}{2}$ is the pyruvate output from muscle (mM/g/min) divided by 2

$\frac{A}{2}$ is the de novo alanine output from muscle (mM/g/min) divided by 2

6 is a constant based on the assumption that 6 millimoles of oxygen are required to oxidize 1 millimole of glucose

$\dot{V}O_2$ is the oxygen consumption of the muscle; converted to mM/g/min (STPD) by the ideal gas law, i.e.,

$$PV = nRT$$

$$n = \frac{(0.92105 \times V)}{(0.08205 \times 310)}$$

100 is a constant based on the conversion of a fraction to a percentage.

In the case of lactate uptake by the muscle, a similar formula was employed

in calculating $\%\dot{V}O_2(CHO)$ with the changes in the formula as follows:

$$\%\dot{V}O_2(CHO) = \frac{[gluc + glyc - (\frac{P}{2} + \frac{A}{2})] \cdot 6 + L.3}{\dot{V}O_2} \cdot 100$$

where:

L.3 is the lactate uptake of the muscle (mM/g/min) multiplied by 3 since 3 millimoles of oxygen are required to oxidize 1 millimole of lactate.

Estimation of the Rate of Turnover of the Citric Acid Cycle. The rate of operation of the citric acid cycle was calculated as follows:

μM of AcCoA/g/min entering CAC

$$= \frac{(\dot{V}O_2) \times \overset{*}{2} \times \overset{**}{2}}{8} \cdot [1 - \frac{\%\dot{V}O_2(CHO)}{100} \cdot \frac{H_o}{H_o + H_i}]$$

where:

$\dot{V}O_2$ is the muscle oxygen uptake expressed as μM/g/min (derived from the ideal gas law)

$\overset{*}{2}$ is the number of oxygen atoms per oxygen mole

$\overset{**}{2}$ is the number of hydrogen equivalents to the number of oxygen atoms

8 is the number of hydrogens produced per "turn of the citric acic cycle"

$\%\dot{V}O_2(CHO)$ is the percentage of the oxygen uptake required for total glucose oxidation

H_o is the number of hydrogens produced from glucose catabolism to 2 moles of acetyl CoA (see example following)

H_i is the number of hydrogens produced from oxidation of 2 moles of AcCoA from glucose through the citric acid cycle (see example following).

The theory supporting estimation of the rate of operation of the citric acid cycle is as follows: if the CAC alone is responsible for all oxidative requirements of the tissue in the M20 group under resting conditions, then

the amount of oxygen required by the CAC and oxidative phosphorylation = 0.37 μM of O_2/g/min (Table 3) or 2 X 0.37 = 0.74 μatoms of oxygen which is equivalent to 2 X 0.74 = 1.48 μatoms of hydrogen oxidized to water in oxidative phosphorylation. Thus $\frac{1.48}{8}$ = 0.18 μmoles of acetyl CoA/g/min must pass into the CAC since in 1 revolution of the cycle, 8 hydrogens are given off. But, it was calculated that 12% of the oxygen uptake of resting muscle in the M20 group was required for total oxidation of glucose ($\%\dot{V}O_2$(CHO) (Table 8). Therefore, one must consider the number of hydrogens produced from glucose catabolism without involvement of the CAC. Consider the relationships between the steps in the oxidation of glucose and the number of hydrogen equivalents of the oxygen used.

Reaction	Number of Hydrogen Equivalents of the Oxygen Used
Glucose ⟶ 1, 3 DPG	4
2 Pyruvate ⟶ 2 AcCoA	4
****	
2 Isocitrate ⟶ 2 αKG	4
2 αKG ⟶ 2 succinate	4
2 Succinate ⟶ 2 malate	4
2 Malate ⟶ 2 OAA	4

Of the 24 hydrogens oxidized to water in glucose oxidation, 8 of these are produced without involving the CAC itself. Therefore, $\frac{1}{3}$ of the 12% of the oxygen consumption was not directly CAC related and the rate of operation of the CAC is overestimated by 4% in the prior calculation which assumed that the CAC alone was responsible for all oxygen requirements of the tissue.

Estimation of the Rate of Turnover of the Malate-Aspartate Shuttle. The rate of operation of the malate-aspartate shuttle system was calculated as follows:

μM of OAA/g/min which are reduced to malate

= 2 · (gluc + glyc) - L

where:

gluc is the glucose arterial-venous difference of the muscle in µM/g/min

glyc is the average rate of glycogen depletion in µM of glucosyl units/g/min

L is the lactate output of the muscle in µM/g/min

2 is a constant: assuming that all glucose uptake and glycosyl units from glycogen are metabolized to pyruvate, then 2 reductions of NAD^+ must occur as a result of the 2 triose molecules produced in the Embden-Meyerhof pathway.

In the case of lactate uptake by the muscle, a similar formula was employed in calculating the rate of operation of the malate-asparate shuttle system with a change in the formula as follows:

µM of OAA/g/min which are reduced to malate

= 2 · (gluc +glyc) + L

where:

L is the lactate uptake of the muscle in µM/g/min.

STATISTICS. Viability and blood dynamic parameters were examined within groups employing 2 two-factor analyses of variance with a repeated measure on 1 factor design. The design of the analyses took the following general form:

	REST	15 min	20 min	40 min	60 min	65 min
Groups M65	X	X	X	X	X	XX
S65	X	X	X	X	X	XX
M20	X	X	XX			
S20	X	X	XX			
analysis 1	*	*	*			
analysis 2	-	-	-			

X: observations of all parameters

XX: observations of selected blood dynamic and viability parameters.

This statistical program is documented with the Division of Educational Research Services (DERS), Computer Program Documentation IBM 360/67, University of Alberta as Fortran IV (H); DERS:ANOV23 (June 1968, revised July 1969) (Winer, 1971, pp. 518). Critical F statistics were evaluated for $p < 0.05$ and a posteriori tests of group means were conducted employing the Scheffé procedure for all possible comparisons (Winer, 1971, pp. 198).

Tissue concentration parameters were treated with 1 way analyses of variance between groups for all right resting muscle data and further 1 way analyses of variance between groups for all left exercised muscle data. This statistical program is documented as DERS:ANOV15 (June 1968, revised July 1969) (Winer, 1971, pp. 152). The Scheffé a posteriori procedure was conducted for all F statistics where $p < 0.05$.

Left muscle concentration parameters were compared with right muscle concentration parameters within each group employing t-tests for correlated samples; DERS:ANOV12 (June 1968, revised July 1969) (Ferguson, 1966, pp. 169-171, 183-184).

Three symmetric matrices of Pearson product-moment correlation coefficients were generated for tissue concentrations, arterial substrate/metabolite concentrations, substrate/metabolite arterial-venous differences, blood flow, systolic and diastolic blood pressure and arterial hemoglobin, hematrocrit and plasma protein concentrations. The separate matrices examined resting conditions, 20 min of stimulation and 65 min of stimulation. The correlation coefficients and the values and prob-

ability of t-tests for each correlation coefficient were produced by the computer program DERS:DEST02 (March 1968, revised July 1969) (Ferguson, 1966, pp. 106-115).

RESULTS

GROUP CHARACTERISTICS. The age, sex, weight, predominant breed characteristic and randomly assigned experimental condition of individual dogs are presented in Appendix VI, Table 1. Group mean values for age and weight of the animals are summarized in Table 1. No differences ($p > 0.05$) were found between group means for age or weight of the animals.

EXPERIMENTAL VIABILITY. The data in Appendix VII, Tables 1-6, represent the effects of sampling and intensity-duration combinations on the physiological and biochemical status of the groups during experimental intervals.

Arterial Metabolite Concentrations. No changes ($p > 0.05$) were noted between pre-exercise resting mean values and subsequent mean values in all groups for femoral arterial concentrations of glucose, pyruvate, lactate, free fatty acids, alanine, lysine, glutamate and aspartate (Appendix VII, Tables 1 and 2).

Arterial Hemoglobin, Hematocrit and Plasma Protein Concentration. The group mean data in Appendix VII, Table 3 indicated that although no alterations ($p > 0.05$) in femoral arterial Hb and Hct existed between resting and final measurements in any group, a decrease ($p < 0.05$) in mean femoral arterial plasma protein concentration was notable between resting and final measurements in group M65.

Arterial and Venous PO_2 and pH. With the exception of acidotic shift ($p < 0.05$) in femoral arterial pH in group S65 between the resting and 60 min sample (7.33 ± 0.01 to 7.29 ± 0.01), no changes ($p > 0.05$)

GROUP	AGE (yr)	WEIGHT (kg)
M20	2.83 (0.83)	21.95 (1.21)
M65	2.83 (0.83)	21.51 (0.42)
S20	2.17 (0.44)	20.35 (1.09)
S65	2.47 (1.03)	23.45 (1.71)

TABLE 1 Age and weight of the experimental animals: Mean (SEM), n = 6

occurred in group means for femoral arterial PO_2 and pH during the experimental period (Appendix VII, Table 4).

Group means for femoral venous PO_2 from rest throughout the contraction period were altered as expected in meeting the two metabolic demands. The lowest mean P_VO_2 values consistently occurred in the S20 and S65 groups with mean values during exercise occurring in the 24.13 to 25.67 mmHg range as compared to 39.98 to 44.53 mmHg at rest in these groups (Appendix VII, Table 4).

Carotid Blood Pressure and Rectal and Muscle Compartment Temperature. Trends in mean carotid arterial blood pressure (Appendix VII, Table 5) and rectal and muscle compartment temperature (Appendix VII, Table 6) revealed no changes ($p > 0.05$) in any group from rest throughout the experiments. However, mean values of resting carotid arterial systolic pressure and resting carotid arterial diastolic pressure were recorded in the ranges of 84.50 to 93.50 mmHg and 46.33 to 60.83 mmHg, respectively.

Deletion of Experiments. Negative viability assessments resulted in the deletion of 2 dogs from the statistical evaluations. In a rejected S65 dog, a blood clot occluded the flow system between 60 and 65 min. A rejected S20 dog demonstrated unusually elevated Hb, Hct and plasma protein concentration. Further, radical hemolysis was noted in this dog in all arterial and venous samples in spite of attempts to avoid stasis in blood sample collection. These rejections resulted in the present n of 6 in each of the 4 groups.

WORK PERFORMANCE. Pre-set resting tensions were similar ($p > 0.05$) for all groups with mean (SEM) values being 1.36 Kg (0.11), 1.50 Kg (0.18), 1.65 Kg (0.12) and 1.47 Kg (0.10) for the M20, M65, S20 and S65 groups, respectively.

Figure 4a reveals mean percentage changes relative to initial tension at each sample interval. This same figure also illustrates the average times required for successive 10 percent decrements in tension during the first 15 min of contraction. Figure 4b demonstrates absolute tension changes from initial through final recordings.

Severe stimulation resulted in greater absolute tension development initially, followed by greater absolute and percentage decrements through subsequent sample intervals (Figure 4a, b). A tension plateau phenomenon was observed with both stimulus intensities since initial large decrements in observed tension occurred prior to 15 min and subsequent declines in tension were far less pronounced.

HISTOCHEMISTRY. Representative colour micrographs of the medial head of gastrocnemius muscle with NADH-diaphorase, PAS and myosin ATPase stains from serial sections of resting muscle and muscle subjected to the 4 intensity-duration combinations are demonstrated in Appendix VIII.

NADH-Diaphorase Staining. The oxidative enzyme stain revealed a positive response in all fibers. Large diameter fibers tended toward dense sub-sarcolemmal formazan granule deposits shading to lighter central staining. Smaller diameter fibers were uniformly darkly stained throughout.

Myosin ATPase Staining. The percentage of fast twitch fibers as determined with the myosin ATPase stain, ranged from mean values of 48.75 to 61.13% between groups with a grand mean of 54.03% for all dogs (Table 2).

As a result of the high oxidative characteristic of all fibers, it was difficult to conclusively distinguish the 3 distinct fiber populations as per Peter et al. (1972), these being slow oxidative (SO), fast glycolytic (FG) and fast oxidative, glycolytic (FOG). It was therefore

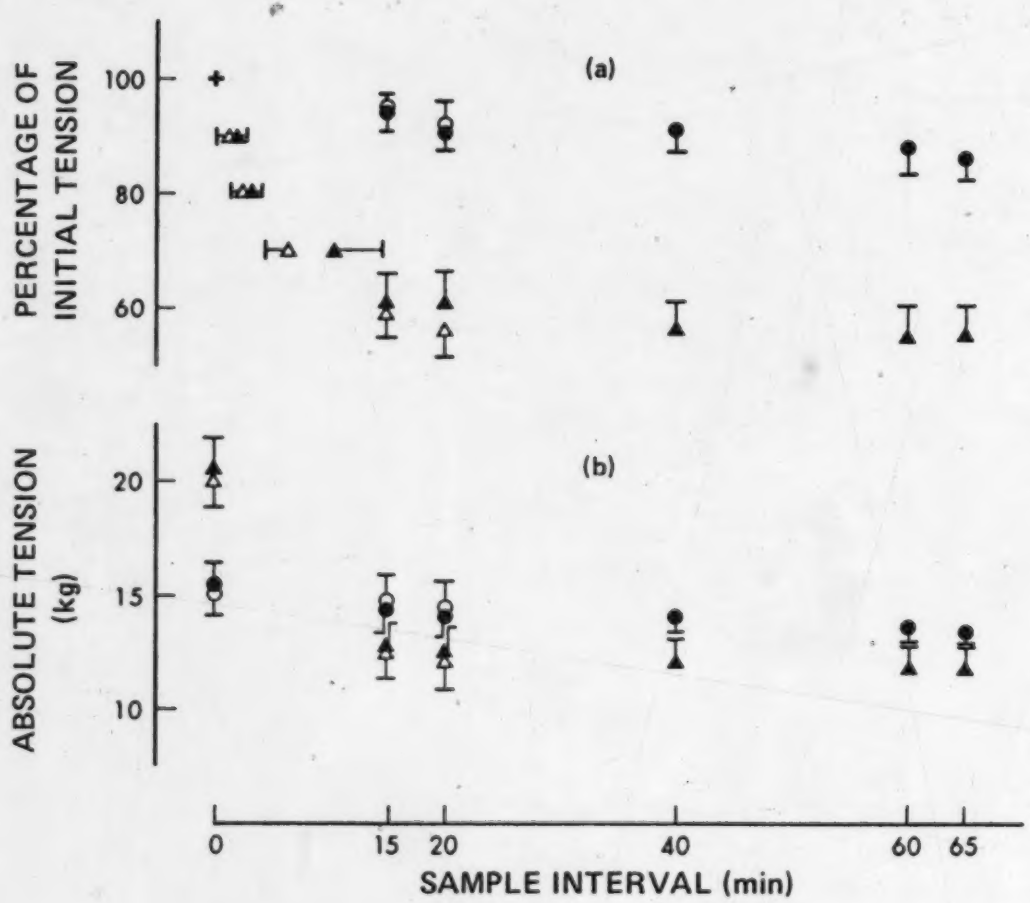


Figure 4 Tension development changes with time as percentages of initial tension (a) and absolute tension development (b) in the specified intensity-duration combinations (○M20; ●M65; △S20; ▲S65)

PARAMETER	GROUP	AVERAGE OF RIGHT AND LEFT GASTROCNEMIUS MUSCLES
% Fast Twitch Fibres Comprising The Muscle	M20	61.13 (2.11)
	M65	48.74 (3.78)
	S20	57.34 (5.44)
	S65	52.83 (1.95)
	GRAND MEAN	54.03 (1.92)

TABLE 2 Group mean values [Mean (SEM), n=6] and grand mean values [Mean (SEM), n=24] for the percentage of fast twitch fibres comprising the left and right gastrocnemius muscles (medial head)

decided to classify all fibers only on their twitch characteristic as indicated by staining intensity for myosin ATPase at pH 9.4.

Periodic Acid-Schiff Staining. The relative PAS stain intensity, as determined subjectively, revealed fast twitch fibers had consistently higher glycogen concentrations under resting conditions. However, with exercise there was a distinct trend toward a more homogeneous PAS staining intensity for glycogen in fast and slow twitch fibers, the rate of which appeared to be directly related to the intensity and/or duration of contraction.

In the M20 group, all fibers were subjectively less dark on the PAS stain than resting muscle. However, fast twitch fibers in M20 were still more intensely stained than the slow twitch fibers in resting tissue. The M65 group demonstrated a trend similar to the M20 group, except that all fibers were lighter. Fast twitch fibers in the M65 group were notably similar to slow twitch fibers under resting conditions on the PAS staining intensity. Severe stimulation resulted in rapid loss of PAS staining intensity such that in the S20 group, slow twitch fibers were very light or showed a negative response to the PAS stain. Fast twitch fibers were only slightly more sensitive to the stain. The S65 group showed a negative PAS response which should reflect very low glycogen concentrations in these tissues.

BLOOD-MUSCLE EXCHANGE DYNAMICS. Blood-muscle exchange dynamics at rest and at the specified sample intervals during contraction are summarized in Tables 3, 4 and 5.

Blood Flow and Oxygen Uptake. Muscle blood flow and oxygen uptake in both exercise intensity groups (M and S) were increased ($p < 0.05$) from resting values at all sample intervals (Table 3). There was a tendency,

not significant ($p > 0.05$), for both blood flow and oxygen uptake to decrease in any one group after 15 min through to the end of experimentation. The mild stimulation groups averaged a 2.8 fold increase over rest in blood flow and 5.2 fold increase over rest in oxygen uptake at 15 min. Severe stimulation likewise produced a 2.8 fold increase above rest in blood flow at 15 min, but oxygen uptake was 7.9 fold over rest at the same sample interval. In the prolonged stimulation groups (M65 and S65), 60 min of mild stimulation resulted in 2.5 fold and 4.1 fold increases above rest in blood flow and oxygen uptake, respectively, whereas, 60 min of severe stimulation resulted in 2.9 fold and 6.3 fold increases above rest in the same respective parameters.

Differences ($p < 0.05$) in blood flow between intensity groups were noted only at 40, 60, and 65 min (S65> M65, Table 3). Oxygen uptake differed ($p < 0.05$) between contraction intensity groups in the following cases: M65 < S20 at 15 min and M65 < S65 at 15, 20, 40 and 60 min (Table 3).

Glucose Uptake. Under resting conditions a small glucose uptake was observed in the muscle preparation (Table 4). Fifteen min of contraction did not result in a change ($p > 0.05$) in the glucose uptake from resting mean values in any one group, even though 1.7 fold and 2.5 fold increases above corresponding resting mean values occurred in the mild and severe stimulus groups, respectively (Table 4). Thereafter, the glucose arterial-venous differences were unaltered ($p > 0.05$) with the exception of the 20 and 60 min glucose arterial-venous differences in the S65 group with were found to be greater ($p < 0.05$) than the corresponding resting mean value.

Differences ($p < 0.05$) between prolonged stimulus severity groups

PARAMETER	GROUP	SAMPLE INTERVAL					
		REST	15 min	20 min	40 min	60 min	65 min
Blood Flow (ml/min)	M20	16.80 (3.08)	49.43 (4.32)*	41.56 (4.00)*			
	M65	16.12 (2.05)	43.33 (7.70)*	41.80 (9.22)*	39.15 (4.31)*	40.24 (5.31)*	35.86 (4.74)*
	S20	20.70 (2.06)	61.08 (8.76)*	62.05 (9.33)*			
	S65	20.12 (2.71)	54.79 (5.77)*	53.02 (3.87)*	59.16 (3.96)*	59.29 (4.32)*	59.07 (4.40)*
Oxygen Uptake (ul/g/min)	M20	10.27 (1.71)	56.19 (8.29)*				
	M65	8.59 (0.95)	40.98 (5.51)*	39.09 (7.49)*	35.88 (2.73)*	35.51 (4.49)*	
	S20	11.02 (1.56)	96.03 (13.60)*				
	S65	10.91 (1.92)	76.37 (10.85)*	76.71 (8.08)*	72.16 (8.41)*	68.91 (7.64)*	

TABLE 3 Blood flow and oxygen uptake at the specified sample intervals: MEAN (SEM), n=6

* significantly different from the corresponding resting value ($p < 0.05$).

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	20 min	40 min	60 min
Glucose A-V Difference (uM/g/min)	M20	0.09 (0.03)	0.18 (0.08)			
	M65	0.09 (0.02)	0.13 (0.09)	0.10 (0.03)	0.12 (0.04)	0.07 (0.03)
	S20	0.11 (0.03)	0.26 (0.09)			
	S65	0.11 (0.02)	0.29 (0.10)	0.32 (0.08)*	0.22 (0.05)	0.21 (0.03)*
Pyruvate A-V Difference (uM/g/min)	M20	-0.0010 (0.0010)	-0.0052 (0.0028)			
	M65	-0.0017 (0.0011)	-0.0113 (0.0107)	-0.0019 (0.0033)	-0.0044 (0.0042)	-0.0026 (0.0013)
	S20	-0.0022 (0.0031)	-0.0427 (0.0109)			
	S65	-0.0013 (0.0017)	-0.0372 (0.0102)	-0.0304 (0.0069)	-0.0284 (0.0086)	-0.0218 (0.0099)
Lactate A-V Difference (uM/g/min)	M20	-0.03 (0.01)	-0.06 (0.03)			
	M65	-0.02 (0.03)	-0.02 (0.04)	0.00 (0.07)	-0.01 (0.03)	0.09 (0.07)
	S20	-0.06 (0.03)	-0.28 (0.04)*			
	S65	-0.07 (0.02)	-0.56 (0.14)*	-0.50 (0.10)*	-0.16 (0.06)	-0.06 (0.06)
Free Fatty Acids A-V Difference (uEq/g/min)	M20	-0.17 (0.02)	0.40 (0.02)*			
	M65	-0.16 (0.01)	0.37 (0.01)*	0.41 (0.02)*	0.83 (0.04)*	0.41 (0.03)*
	S20	-0.14 (0.01)	0.75 (0.05)*			
	S65	-0.20 (0.02)	0.85 (0.01)*	0.64 (0.03)*	0.77 (0.03)*	0.54 (0.02)*

con't.

PARAMETER	GROUP	SAMPLE INTERVAL				
		REST	15 min	20 min	40 min	60 min
Plasma Water Loss (ul/g/min)	M20	-2.12 (1.75)	-10.09 (8.06)			
	M65	-5.94 (1.05)	- 4.52 (2.84)	-10.77 (5.22)	-2.95 (2.03)	-1.31 (2.05)
	S20	-0.95 (3.37)	-10.01 (6.82)			
	S65	-6.21 (1.51)	- 4.25 (2.72)	- 4.80 (6.25)	1.81 (14.04)	7.52 (10.76)

TABLE 4 Metabolite arterial-venous differences and plasma water loss at the specified sample intervals: MEAN (SEM), n=6.

* significantly different from the corresponding resting values ($p < 0.05$).

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	60 min
Alanine A-V	M20	-10.81 (3.96)	-161.82 (32.90)*	-145.15 (57.54)*
Difference	M65	-14.54 (3.96)		-145.15 (57.54)*
(nM/g/min)	S20	-24.45 (16.33)	-212.02 (86.59)*	
	S65	-20.45 (8.34)		-122.73 (33.21)*
Lysine A-V	M20	- 7.90 (1.54)	- 30.27 (7.16)*	
Difference	M65	- 5.57 (0.76)		- 27.85 (4.40)*
(nM/g/min)	S20	- 7.87 (5.26)	- 33.21 (17.30)	
	S65	- 5.40 (1.35)		- 23.63 (11.04)
Glutamate A-V	M20	1.42 (1.87)	- 0.77 (6.14)	
Difference	M65	- 3.03 (3.67)		- 5.49 (3.27)
(nM/g/min)	S20	2.29 (1.80)	- 47.85 (26.55)	
	S65	6.39 (8.58)		- 11.18 (10.46)
Aspartate A-V	M20	0.44 (0.23)	0.69 (1.09)	
Difference	M65	0.81 (0.31)		- 0.17 (0.46)
(nM/g/min)	S20	0.63 (0.37)	- 2.90 (1.63)	
	S65	1.36 (0.24)		- 1.05 (0.98)*

TABLE 5 Arterial-venous differences of the selected amino acids at the specified sample intervals: MEAN (SEM), n=6.

* significantly different from the corresponding resting value ($p < 0.05$).

(M65 and S65) occurred at 20 and 60 min in that the S65 group demonstrated a greater glucose uptake at these time intervals (Table 4).

Pyruvate Output. The muscle preparation showed slight pyruvate output under resting conditions (Table 4). Mild or severe stimulation resulted in no changes ($p > 0.05$) from resting output mean values at any sample interval. However, pyruvate output in the M20 group was less ($p < 0.05$) than that of the S20 and S65 groups at 15 min, while the M65 group showed less ($p < 0.05$) pyruvate output than the S65 group at 20 min (Table 4).

Lactate Output. Lactate output from the resting muscle was observed (Table 4). Exercise resulted in no change ($p > 0.05$) from rest values in lactate output in the mild stimulus group at 15 min. Severe stimulation, however, resulted in a greater ($p < 0.05$) lactate output at 15 and 20 min when compared to corresponding resting values. After the above mentioned sample intervals, exercise lactate arterial-venous differences decreased, though not significantly ($p > 0.05$), such that lactate uptake was observed at 60 min in the M65 group while lactate output was not substantially different ($p > 0.05$) from rest values in the S65 group.

Differences ($p < 0.05$) in lactate arterial-venous differences between intensity groups occurred only in exercise at 15 and 20 min (Table 4).

FFA Output. A consistent, small free fatty acid output from the muscle preparation was observed in all groups at rest under present experimental conditions (Table 4). Electrical stimulation of the mild or severe nature produced a free fatty acid uptake ($p < 0.05$) in all groups over all sample intervals as compared to resting outputs. Differences ($p > 0.05$) between severity groups, however, were not noted (Table 4).

The muscle preparation took up small amounts of plasma water at rest (Table 4). No changes ($p > 0.05$) were observed in plasma water loss in comparisons of duration or intensity effects (Table 4).

The muscle preparation put out alanine and lysine and accumulated small amounts of glutamate and aspartate under resting conditions (Table 5).

Alanine Output. Alanine output by the muscle increased ($p < 0.05$) dramatically in both M and S groups at 15 min (15.0 fold and 8.7 fold above rest, respectively) (Table 5). This output decreased to smaller, but significant ($p < 0.05$), arterial-venous differences at 60 min (9.9 fold and 6.0 fold above rest in the M65 and S65 groups, respectively. The differences between severity groups at each sample interval for the arterial-venous different of alanine were not significant ($p > 0.05$).

Lysine Output. Lysine output followed a similar trend as alanine, though much reduced in absolute quantities, in that alanine output exceeded lysine output by approximately 5 to 6 fold during exercise of either intensity (Table 5).

Aspartate and Glutamate Output. Both aspartate and glutamate arterial-venous differences during exercise were highly variable in that some muscle preparation accumulated these amino acids whereas others released them under similar conditions (Table 5). Only aspartate output after 60 min of contraction was found to be different ($p < 0.05$) from the corresponding group mean resting value. No differences ($p > 0.05$) were established in either of the glutamate or aspartate arterial-venous differences when contraction intensity groups were compared at the specified sample intervals.

MUSCLE TISSUE CONCENTRATIONS. Group mean values for the right and left muscle weights are presented in Table 6. Differences between intensity-

PARAMETER	GROUP	RIGHT RESTING MUSCLE	LEFT STIMULATED MUSCLE
Gastrocnemius Muscle Wet Weight	M20	82.28 (3.15)	87.85 (4.53)
	M65	90.28 (2.43)	91.95 (2.78)
	S20	82.30 (6.12)	82.35 (6.63)
	S65	97.88 (7.45)	103.40 (8.26)

TABLE 6 Wet weight of the right non-stimulated and left stimulated gastrocnemius muscles: Mean (SEM), n=6

PARAMETER	GROUP	RIGHT MUSCLE NON-STIMULATED	LEFT MUSCLE 20 MIN STIMULATION	LEFT MUSCLE 65 MIN STIMULATION	LEFT/ RIGHT RATIO
Glycogen	M20	20.23 (2.53)	16.78 (2.09)		0.83
(μM glycosyl	M65	35.02 (4.96)		26.14 (4.91)	0.75
units/g wet wt)	S20	18.11 (2.81)	10.55 (3.06)		0.58
	S65	15.71 (1.75)		6.17 (0.89)*	0.39
Triglycerides	M20	29.33 (11.44)	10.96 (4.64)		0.37
(μM tripalmi-	M65	7.24 (2.10)		7.13 (1.61)	0.98
tin/g wet wt)	S20	22.99 (13.11)	10.18 (2.76)		0.44
	S65	9.46 (2.26)		7.13 (0.76)	0.75
Alanine	M20	2.43 (0.09)	1.68 (0.09)		0.69
(μM/g wet wt)	M65	2.75 (0.18)		1.52 (0.20)*	0.55
	S20	2.78 (0.39)	2.63 (0.26)		0.95
	S65	2.76 (0.23)		1.83 (0.46)	0.66
Lysine	M20	0.54 (0.08)	0.40 (0.04)		0.75
(μM/g wet wt)	M65	0.48 (0.07)		0.34 (0.08)	0.72
	S20	0.52 (0.11)	0.30 (0.04)		0.58
	S65	0.43 (0.07)		0.23 (0.03)	0.54
Glutamate	M20	4.48 (0.24)	2.47 (0.27)		0.55
(μM/g wet wt)	M65	4.07 (0.26)		2.97 (0.24)	0.73
	S20	3.77 (0.34)	0.99 (0.07)		0.26
	S65	3.66 (0.46)		0.94 (0.10)	0.26
Aspartate	M20	0.42 (0.03)	1.19 (0.26)		2.80
(μM/g wet wt)	M65	0.41 (0.03)		0.68 (0.08)	1.64
	S20	0.43 (0.06)	0.66 (0.21)		1.54
	S65	0.40 (0.06)		0.65 (0.12)	1.63

TABLE 7 Muscle metabolite concentrations in the right non-stimulated and left stimulated muscles and the left to right ratio for the specified sample intervals: MEAN (SEM), n=6.

* significantly different from the corresponding resting mean value ($p < 0.05$).

duration groups were not significant ($p > 0.05$). A summary of glycogen, triglyceride, alanine, lysine, aspartate and glutamate concentrations in right non-stimulated muscle and left exercised muscle for the four intensity-duration groups as well as left versus right ratios for these variables are reported in Table 7.

Right non-stimulated muscle glycogen concentrations were higher ($p < 0.05$) in the M65 group when compared to the other 3 groups (Table 7). In spite of these initially different resting levels, a consistent trend in left to right ratios was established with relative glycogen depletion occurring at a rate affected by both stimulus intensity and duration. Examination of absolute concentration differences between right and left muscle revealed the average rate of glycogen depletion between initiation (rest) and 20 min of stimulation in the S20 group was 2.2 fold greater than the rate in the M20 group (0.38 and 0.17) μmoles of glycosyl units/g/min, respectively). However, the average rates of glycogen breakdown between 20 and 65 min were 0.065 μmoles of glucosyl units/g/min in both the mild and severe stimulus groups in spite of the noted differences in the left exercise to right resting muscle ratios (0.75 and 0.39) for the M65 and S65 groups, respectively).

The data for muscle triglyceride concentrations in the pre-exercise muscle preparation revealed no differences ($p > 0.05$) among groups. (Table 7). Combinations of intensity and duration of muscle contraction did not alter ($p > 0.05$) the concentration of this stored energy source.

Intramuscular concentrations of the free amino acids, alanine, lysine, glutamate and aspartate showed little variability ($p > 0.05$) between groups under resting conditions (Table 7). Of these four amino acids, only the free alanine concentration in muscle was altered ($p < 0.05$) by stimulus duration and this was confined to a decrease ($p < 0.05$) from

resting concentration in the M65 group at 65 min.

Comparison of stimulus intensity effects on free amino acid concentrations, assessed from absolute concentrations, revealed that differences were confined primarily to alanine and glutamate concentrations (Table 7). Alanine concentration was greater ($p < 0.05$) in the S20 group when compared to the M20 group at 20 min. There was no difference ($p > 0.05$) in alanine concentration at 65 min. Glutamate concentration was less ($p < 0.05$) in the S20 group as compared to the M20 group at 20 min and also less ($p < 0.05$) in the S65 group compared to the M65 group at 65 min.

The trends in left to right ratios of the specified free amino acids reveal that, in general, mild stimulation produced a decrease in tissue alanine, lysine and glutamate concentrations and a marked increase in the tissue aspartate concentration (Table 7). Severe stimulation resulted in a smaller decrease in the tissue alanine concentration, greater decreases in the glutamate and lysine concentrations and a smaller increase in the aspartate concentration than did the mild stimulus. Twenty minutes of mild and severe stimulation, respectively, resulted in 31 and 5% decreases in alanine, 25 and 42% decreases in lysine, 45 and 74% decreases in glutamate and 180 and 54% increases in aspartate. Between 20 and 65 min of stimulation, the alanine left to right ratio decreased a further 14% in mild stimulation and 29% in severe stimulation. During this same time interval, tissue lysine was unchanged in that the left to right ratios were altered beyond 20 min by only 3 to 4%. Tissue glutamate showed no change in left to right ratios in the S65 group (0.26) compared to the S20 group (0.26). However, in the mild stimulus groups, left to right ratios for glutamate tended to increase in the S65 group (0.73) compared to the S20 group (0.55) though it did not return to resting concentrations. Tissue aspartate was not affected by continuance of severe stimulation after 20 min. Left to right ratios for this amino

acid differed by only 9% between 20 and 65 min, respectively. On the other hand, mild stimulation resulted in a large decrease in the left to right ratios for aspartate between 20 and 65 min. After the initial 2.8 fold increase in aspartate at 20 min of mild stimulation, its concentration decreased at 65 min to only 0.64 fold over the original resting value.

THE PERCENTAGE OF THE OXYGEN UPTAKE OF THE MUSCLE ACCOUNTED FOR IN CARBOHYDRATE OXIDATION. Estimates of the $\%\dot{V}O_2(CHO)$ under resting and exercise conditions for each intensity-duration group are demonstrated in Table 8.

Under resting conditions, the muscle preparation required 10 to 14% of its $\dot{V}O_2$ for oxidation of the glucose taken up (i.e., where glycogen catabolism at rest was assumed to be zero). Mild stimulation of the muscle increased this proportion to 76% at 15 min. A subsequent decline from this value was then noted as only 58% of the oxygen uptake was needed to oxidize glucose and glycogen through the CAC after 60 min of mild effort. Severe stimulation resulted in slightly lower $\%\dot{V}O_2(CHO)$ than did mild stimulation. Carbohydrate oxidation required 62 and 44% of the muscle oxygen uptake at 15 and 60 min in the S20 and S65 groups, respectively.

ESTIMATES OF THE QUANTITY OF α-KETOGLUTARATE GENERATED FROM GLUTAMATE PYRUVATE TRANSAMINATION. Based on the stoichiometry of the glutamate-pyruvate transamination reaction, it was estimated that <u>de novo</u> synthesis of αKG from glutamate-pyruvate transamination accounted for 0.003 to 0.017 μmoles of αKG/g/min in the muscle preparation under resting conditions (Table 9). Exercise conditions increased the estimated <u>de novo</u> synthesis of αKG from glutamate-pyruvate transamination at 15 min in both stimulus intensities (46 and 11 fold above rest in M20 and S20, respectively). A decline in αKG synthesis from this source was noted at 60 min of stimulation relative to estimated αKG production at 15 min in both stimulus intensities (13 and 7 fold above rest in M65 and S65, respectively).

MUSCLE REQUIREMENTS FOR CITRIC ACID CYCLE INTERMEDIATES. The estimated flux rates though the citric acid cycle and the malate aspartate shuttle at rest and during each of the intensity-duration combinations of exercise are summarized in Figure 5. The proportions of these fluxes that could be accounted for by αKG produced in the glutamate-pyruvate transamination reaction are summarized as individualized estimates in Figure 6 (i.e., estimate assumes all αKG from glutamate-pyruvate transamination acted to satisfy either one or the other flux, but not a combined requirement). Figure 7 provides an estimate of the total requirement of the muscle preparation for OAA at rest or during exercise, based on the sum of estimated fluxes in the CAC and in the malate-aspartate shuttle. The estimated proportion of the total need of the muscle for OAA accounted for by αKG from the glutamate-pyruvate transamination reaction is also presented in Figure 7.

Both mild and severe stimulation increased the individual pathway and total requirements for OAA in the muscle preparation above resting levels, with severe stimulation imposing the greater demand at both the 15 and 60 min intervals (Figures 5 and 7). The estimated requirements for OAA in the CAC was greater than that in the malate-aspartate shuttle for all intensity-duration combinations (Figure 5). A marked decline in OAA requirement between 15 and 60 min was noted in both mild and severe stimulation.

Estimation of the contribution of αKG from glutamate-pyruvate transamination in satisfying the needs of the individual pathways or the total muscle implied that during mild stimulation, glutamate-pyruvate transamination activity satisfied a greater proportion of metabolic needs for OAA at both the 15 and 60 min sample intervals. The greatest potential

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	60 min
Oxygen Uptake (µl/g/min)	M20	10.27 (1.71)	56.19 (8.29)	
	M65	8.59 (0.95)		35.51 (4.49)
	S20	11.02 (1.56)	96.03 (13.60)	
	S65	10.91 (1.92)		68.91 (7.64)
% VO_2 (CHO)	M20	12	76	
	M65	14		58
	S20	11	62	
	S65	10		44

TABLE 8 Estimated percentage of the muscle oxygen uptake accounted for in carbohydrate metabolism at rest and after specified contraction intervals

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	60 min
Estimated μM of α KG/g/min from GPT	M20	0.003	0.126	
	M65	0.009		0.111
	S20	0.017	0.230	
	S65	0.015		0.111

TABLE 9 Estimated production of α-ketoglutarate from glutamate-pyruvate transamination at rest and after specified contraction intervals

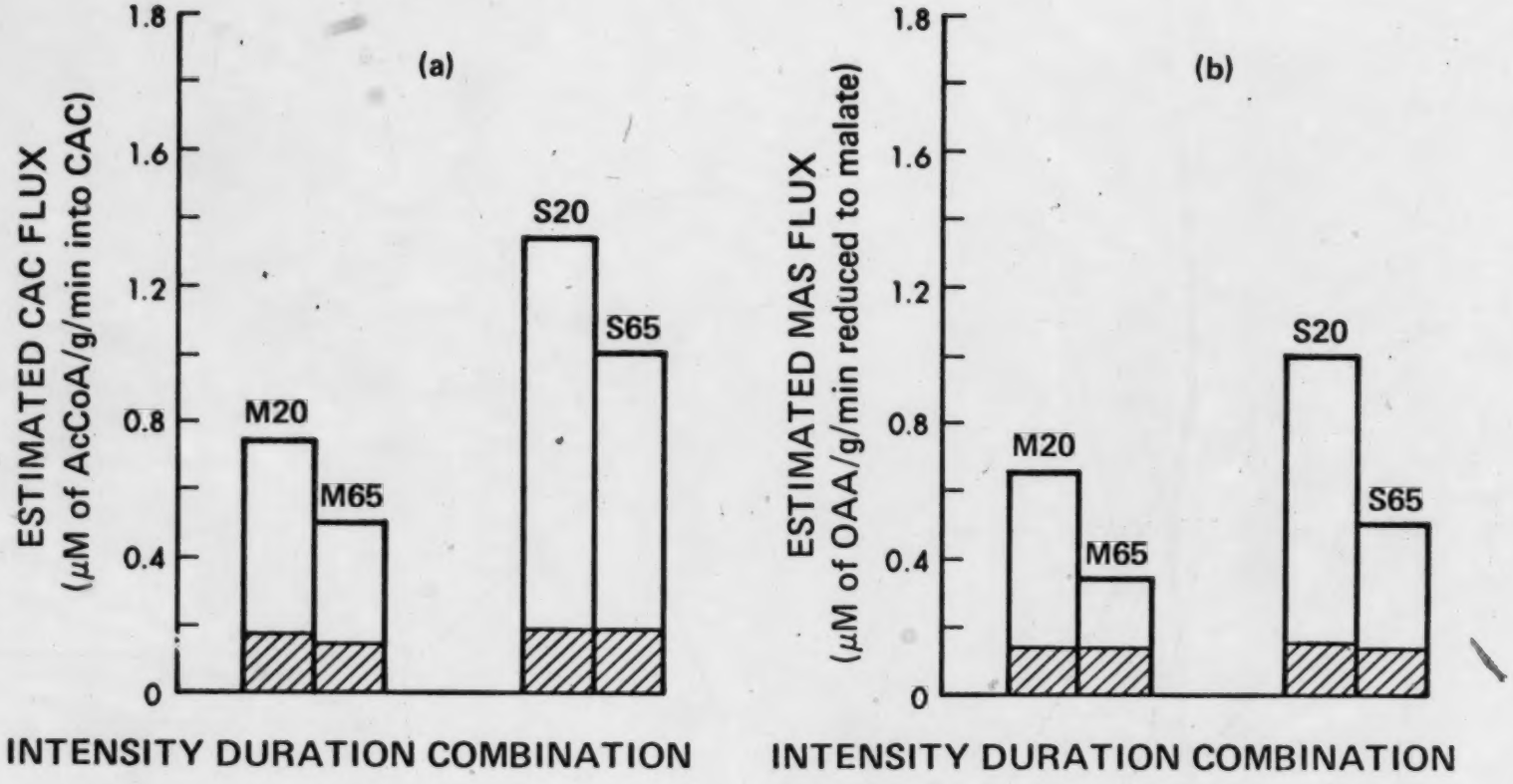


Figure 5 Estimated turnover rates of the citric acid cycle (a) and the malate-aspartate shuttle (b) in the specified intensity-duration combinations (hatched areas represent resting conditions)

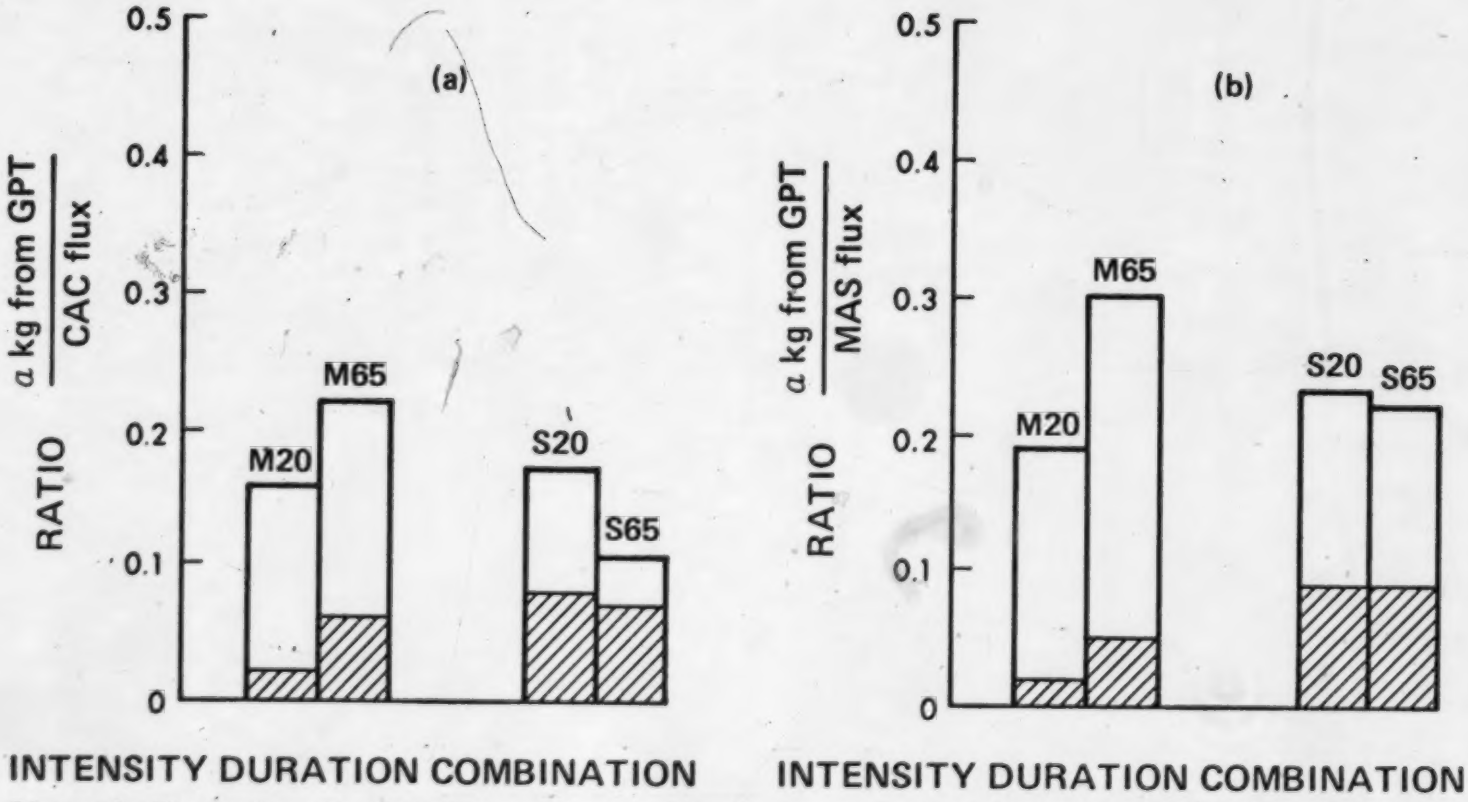


Figure 6 The ratios of α-ketoglutarate production from glutamate-pyruvate transamination to citric acid cycle flux (a) and malate-aspartate shuttle flux (b) in the specified intensity-duration combinations (hatched areas represent resting conditions)

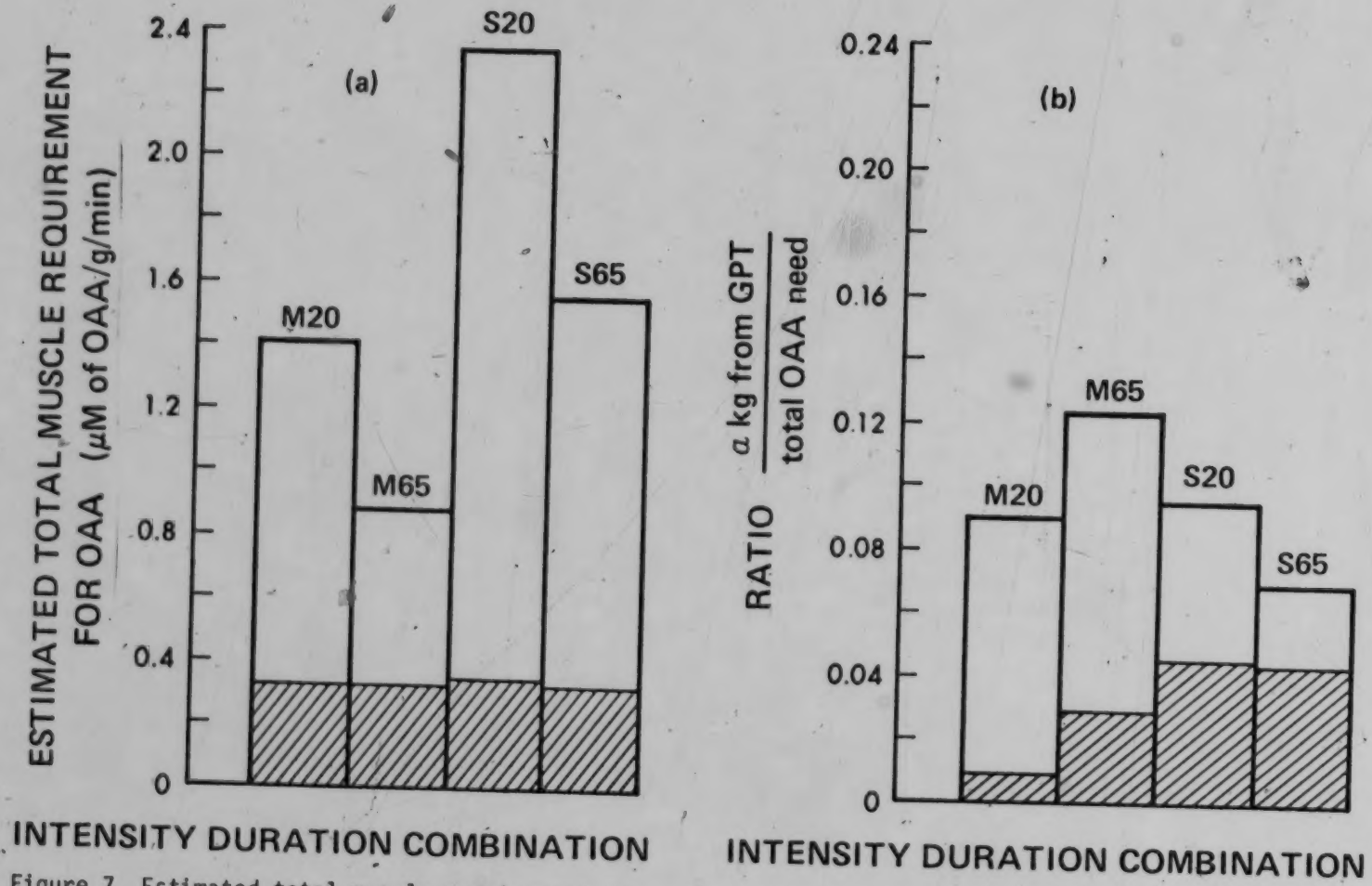


Figure 7 Estimated total muscle requirement for oxaloacetate turnover (a) and the ratio of α-ketoglutarate production from glutamate-pyruvate transamination to total oxaloacetate flux (b) in the specified intensity-duration combinations (hatched areas represent resting conditions)

contribution of αKG from glutamate-pyruvate transamination to the total tissue requirement for OAA flux was 12% in the M65 group.

CORRELATIONS BETWEEN MUSCLE AMINO ACID DYNAMICS AND SELECTED METABOLIC PARAMETERS. Tables 10, 11 and 12 include the significant ($p < 0.05$) correlations among tissue concentrations of the selected free amino acids, oxygen uptake and the arterial-venous differences of alanine, glutamate, aspartate, pyruvate, lactate and glucose under pre-exercise conditions and after 15 or 60 min of contraction. The entire correlation matrices for these and other experimental parameters are located in Appendix IX.

After 15 min of contraction, the two highest statistically significant relationships were between the lactate arterial-venous difference and the glutamate tissue concentration and arterial-venous difference ($r = 0.83$ and 0.73, respectively, Table 11). Tissue concentrations of alanine were correlated ($p < 0.05$) positively with the oxygen uptake of the muscle ($r = 0.56$) and correlated ($p < 0.05$) negatively with the tissue glutamate concentration ($r = 0.65$) and the arterial-venous differences for lactate and pyruvate ($r = -0.59$ and -0.66, respectively). The alanine output of the muscle was negatively correlated ($p < 0.05$) with the glucose uptake of the muscle ($r = -0.60$) at the same period (Table 11).

Sixty min of adaptation to sustained contractile activity resulted in a high negative correlation ($p < 0.05$) between the tissue concentrations of alanine and aspartate ($r = -0.65$). However a high positive correlation ($p < 0.05$) was established between the alanine and aspartate and arterial-venous differences ($r = 0.64$, Table 12).

CORRELATES (resting conditions)	n	r
Tissue Alanine vs pyruvate A-Vd	24	0.56
Tissue Glutamate vs tissue aspartate	24	0.62
Glutamate A-Vd* vs oxygen uptake	24	0.45
Tissue Aspartate vs oxygen uptake	24	0.46
Aspartate A-Vd* vs glucose A-Vd*	24	0.52

TABLE 10 The significant ($p < 0.05$) correlations between tissue free amino acid concentrations, oxygen uptake and arterial-venous dynamic parameters under resting conditions.

* A-Vd is the arterial venous difference

CORRELATES (15 min of muscle stimulation)	n	r
Tissue Alanine		
vs		
oxygen uptake	12	0.56
tissue glutamate	12	-0.65
pyruvate A-Vd*	12	-0.56
lactate A-Vd*	12	-0.59
Alanine A-Vd*		
vs		
glucose A-Vd*	12	-0.60
Tissue Glutamate		
vs		
oxygen uptake	12	-0.58
pyruvate A-Vd*	12	0.68
lactate A-Vd*	12	0.83
Glutamate A-Vd*		
vs		
aspartate A-Vd*	12	0.63
lactate A-Vd*	12	0.73
Aspartate A-Vd*		
vs		
oxygen uptake	12	-0.62
lactate A-Vd*	12	0.63

TABLE 11 The significant ($p < 0.05$) correlations between tissue free amino acid concentrations, oxygen uptake and arterial-venous dynamic parameters after 15 minutes of muscle stimulation

* A-Vd is the arterial venous difference

CORRELATES (60 min of muscle stimulation)	n	r
Tissue Alanine vs tissue aspartate	12	-0.65
Alanine A-Vd* vs aspartate A-Vd*	12	0.64
Tissue Glutamate vs oxygen uptake	12	-0.63
glucose A-Vd*	12	-0.58

TABLE 12 The significant ($p < 0.05$) correlations between tissue free amino acid concentrations, oxygen uptake and arterial-venous dynamic parameters after 60 minutes of muscle stimulation.

* A-Vd is the arterial venous difference

DISCUSSION

AN EXERCISE FRAME OF REFERENCE. Peter et al. (1972) have stated that for interpretation of biochemical studies of skeletal muscle, selection of muscle of one fiber type is necessary to avoid obfuscation. Therefore, an important step in evaluating the functional significance of present observations was the establishment of a frame of reference for exercise conditions. The following discussion of metabolic interactions pertains specifically to exercise involving complete recruitment of all muscle fibers in a homogeneously high oxidative skeletal muscle (Appendix VIII comprised of equal proportions of fast and slow twitch units (Table 2).

Previous research from this laboratory (Gardiner, 1976) revealed that the initial rapid decline in tension development up to 15 min as seen here, was highly related to lactate accumulation in the muscle preparation presumably as a result of an initial high rate of glycolysis in FOG fibers. The marked decline in lactate production after 5 to 7 min of exercise as reported by Hirche et al. (1970b, 1971, 1973, 1975) and Gardiner (1976) suggested a shift in metabolism from predominantly glycolysis to a more oxidative pattern of metabolism. Hence, this stage of metabolic adaptation to exercise was avoided in selection of the present sample intervals since primary interest lay with the interactions of the specified pathways during sustained, predominantly oxidative energy metabolism.

The use of the in situ gastrocnemius muscle preparation as a model for prolonged metabolic experimentation and the observed trends in ten-

sion development in these studies has been reported elsewhere (Welch and Stainsby, 1967; DiPrampero et al. 1969; Hirche et al., 1970a, b, 1971; Fitts et al., 1973; Morganroth et al., 1975; Horstman et al., 1976). The capacity of the dog gastrocnemius muscle for prolonged, rhythmic tension development (Figure 4) was consistent with the presence of only highly oxidative muscle fibers in this muscle (Appendix VIII) (Maxwell et al., 1975). Even with recruitment of all muscle fibers by direct maximal stimulation, pronounced fatigue, as judged from tension changes, was not seen. It was apparent though, that the maximum tension developed by the muscle at the initiation of exercise could not be maintained. An initial rapid decline in tension development (to 15 min) was followed by a steady state of not less than 50% of the original capacity for tension development even after 1 hour of continual activity.

In spite of similarities between work performance in this and other studies, interpretation of present metabolic data and comparison of these data with others of a similar nature was a complex matter. It was evident that a variety of experimental conditions may limit inferences as to muscle metabolism in such experiments since no standardized pattern of experimental technique has emerged. The following factors were of primary concern in this regard: 1) the extent of interanimal variability (i.e., different breeds of dogs), 2) the choice of anesthetic agent, 3) the degree of viability maintained in the dog as a whole as well as the constancy of the physiological and biochemical milieu of the in situ muscle and 4) the temporal pattern and frequency of stimulation plus the resultant contraction whether it be sustained or rhythmic, isometric or isotonic, tetanic or twitch.

Where possible, rigorous procedures were followed in this study to

control or account for those factors which may have altered the metabolic responses of the anesthetized dog and its gastrocnemius muscle to the rhythmic, isotonic tetanic trains from direct maximal muscle stimulation.

INTERANIMAL VARIABILITY. A number of reports of comparable nature to the present study have indicated large ranges in metabolic responses of muscle which were directly associated with a heterogeneous population of dogs (Stainsby and Welch, 1966; Chapler and Stainsby, 1968; Hirche et al., 1970b; Chapler and Katrusiak, 1974). In addition, Secord and Russell (1973) have compared Labrador retrievers to mongrel dogs on a large number of metabolic and hematological parameters. Selective breeding for stamina in Labrador retrievers was found to result in greater data homogeneity in this population of dogs. Also, differences between Labrador retrievers and mongrels in metabolic patterns and more specifically, significant differences in circulating levels of pyruvate and lactate, were of particular consequence to present studies. Labrador retrievers were characterized by higher circulating lactate but lower circulating pyruvate.

Although these constraints were known at the outset, the use of a small number of mixed breeds of dogs was necessitated in this study by financial restraints. Therefore, attempts were made to "equalize" groups by random assignment without replacement in the case of pure-bred dogs. This design met with partial success in that even though groups appeared to be balanced as to breed occurrence (Appendix VI, Table 1), a high degree of variability was established in several key metabolic parameters (Tables 3, 4, 5 and 7). This interanimal variation may have resulted in failure to demonstrate consistent significant ($p < 0.05$) differences between intensity-duration groups in certain cases.

THE CHOICE OF ANESTHETIC AGENT. It is commonly held that anesthesia constitutes a physiological trespass on the animal and differential alteration of homeostasis by various anesthetics is well documented (Chenoweth and VanDyke, 1969a, b; Miller, 1969; Strobel and Wollman, 1969). Thus, conclusions based on results where only one anesthetic is used can prove misleading.

The primary bases for selection of the anesthetic agent (halothane) in the present study were associated with maintenance of a constant plane of anesthesia and avoidance of some of the critical side-effects of the barbituate, sodium pentobarbital, which is commonly employed in similar studies (Corsi et al., 1969; Barclay and Stainsby, 1972; Whalen et al., 1973; Duran and Renkin, 1974; Morganroth et al., 1975; Horstman et al., 1976). In brief, barbituates produce decreased sympathetic tone, decreased cardiac output, respiratory acidosis and decreased circulating lactate and pyruvate levels (Chenoweth and VanDyke, 1969b; Strobel and Wollman, 1969). More recently, Dunn and Critz (1975) have elaborated on the barbituate induced inhibition of fatty acid oxidation in skeletal muscle (Armstrong et al., 1961) and shown that during stimulation of dog skeletal muscle, this inhibition may produce misleading lactate production data.

Halothane, as an inhalant, readily satisfied present criteria. However, the complications of this choice bear mention. The enhancement of body heat losses through peripheral dilation, decreased respiration, decreased cardiac output and decreases in mean blood pressure have been attributed to this anesthetic (Miller, 1969; Strobel and Wollman, 1969). Direct metabolic effects of halothane have not been widely documented, although Mitchelson and Hird (1973) have found that skeletal muscle mito-

chondria are sensitive to concentrations of halothane greater than 3 millimolar, a much higher tissue concentration than normal anesthesia would produce (Miller, 1969).

MAINTENANCE OF EXPERIMENTAL VIABILITY. Attempts to counteract the physiological phenomenon associated with halothane anesthesia were largely successful in these experiments. Muscle and rectal temperatures were maintained by artificial methods (Appendix VII, Table 6) while femoral P_AO_2 (Appendix VII, Table 4) was also regulated in line with data for unanesthetized dogs (Fiegl and D'Alecy, 1972). However, it was not possible to maintain normal arterial blood pressure in these experiments without artificially perfusing the muscle. Mean values for systolic and diastolic blood pressure (Appendix VII, Table 5) were recorded at subnormal levels (Tipton et al., 1974). The relevance of this observation will be clarified at a later point (pp. 73).

Due to the nature of metabolic experimentation and its related potential for subsequent interpretation, selected physiological and biochemical variables, besides those known to be affected by halothane anesthesia, were considered of importance in providing a normal environment for the working muscle. Comparison of characteristics used to judge viability in this study with values reported for unanesthetized dogs revealed that mean arterial concentrations (Appendix VII, Table 2) were normal for glucose (Paul et al., 1966; Canadian Council on Animal Care), lactate (Paul, 1970), free fatty acids (Paul et al., 1966; Paul, 1970), and the selected free amino acids (Hier and Bergéim, 1946). Comparative data for arterial pyruvate concentration in unanesthetized dogs was unavailable.

Mean values of arterial Hb and Hct (Appendix VII, Table 3) were comparable to accepted canine hematological data (Secord and Russell,

1973; Greenleaf et al., 1974; Tipton et al., 1974; Canadian Council on Animal Care) whereas mean arterial pH values (Appendix VII, Table 4) fell in the low normal range for unanesthetized dogs (Fiegl and D'Alecy, 1972). Mean values for arterial total proteins (Appendix VII, Table 3) were comparable to the resting range in unanesthetized dogs (Greenleaf et al., 1974).

Of those variables considered for viability assessments, only arterial plasma proteins (Appendix VII, Table 3) and arterial pH (Appendix VII, Table 4) demonstrated significant ($p < 0.05$) shifts during prolonged experiments in the M65 and S65 groups, respectively. It should be noted, however, that arterial plasma proteins and arterial pH followed similar, though not significant ($p > 0.05$) trends, in the other prolonged experiments (S65 and M65 groups, respectively).

The decreases in arterial plasma proteins beyond 20 min in the M65 group ($p < 0.05$) and S65 group ($p > 0.05$) most likely reflected the combined effects of surgical blood loss, sampling blood loss and dilution of the remaining pool of blood by the intravenous Ringer's drip. However, the lack of correlation ($p > 0.05$) between this and other exercise experimental parameters (Appendix IX, Table 3 and 4) and the lack of similar decreases ($p > 0.05$) in arterial Hb and Hct (Appendix VII, Table 3) in these groups at these same time periods suggests little metabolic importance relative to this observation.

The shift in femoral arterial pH under prolonged exercise conditions in the M65 group ($p > 0.05$) and the S65 group ($p < 0.05$) (Appendix VII, Table 4) may have been the result of respiratory depression and a related acidosis (Chenoweth and VanDyke, 1969b). Arterial pH values of 7.24 (M65 at 60 min) and 7.29 (S65 at 60 min) fall outside of accepted limits for normal unanesthetized canines (Fiegl and D'Alecy, 1972). However, these

values are in excess of the experimental acidosis induced by Hirche et al. (1975). Blood pH in their experiments (6.95 to 7.10) resulted in decreased release of lactate from dog skeletal muscle, decreased muscle work per unit time and rapid decreases in muscle $\dot{V}O_2$. These observations (Hirche et al., 1975) were believed to reflect higher lactate concentrations in the muscle. In addition, Harken (1976) has discussed the critical dependency of muscle $\dot{V}O_2$ on extracellular fluid pH. It appears that $\dot{V}O_2$ may be altered as much as 10% by pH changes of 0.1. However, data from this laboratory (Gardiner, 1976) does not support metabolic and work performance effects in the in situ dog muscle preparation as a result of the small acidosis seen in the present groups. In fact, the slight pH decreases observed here may have been important in increasing blood flow in exercising in situ muscle. Stowe et al. (1975) have reported that blood pH decreases in exercise are directly associated with exercise hypermia. Artificial increases in pH to resting values in their work (Stowe and coworkers) was accompanied by decreases in blood flow to resting levels, even though the in situ muscle was performing work.

The availability of oxygen to skeletal muscle is affected by blood flow, blood oxygen tension and the number of capillaries participating in gas exchange (Stainsby and Otis, 1964; Hudlicka, 1973; Wenger and Reed, 1976). Therefore the ability of denervated muscle to spontaneously regulate its own blood supply to varied metabolic demands (Hudlicka, 1973) is an important index of viability during long lasting experiments where deterioration of physiological conditions may occur (Barclay and Stainsby, 1975).

The low correlation ($r = -0.04$, $p > 0.05$) between resting blood flow and resting $\dot{V}O_2$ of the muscle in this study indicated autoregulation of resting blood supply. Duran and Renkin (1974) and Barclay and Stainsby

(1975) have reported that resting $\dot{V}O_2$ in dog skeletal muscle preparations was independent of blood flow only if regulatory vascular responses were present in the muscle. On the other hand, parallel shifts in the muscle $\dot{V}O_2$ and its blood flow during exercise sample intervals in the present work (Table 3) also suggested that autoregulation was maintained in exercise conditions (Barclay and Stainsby, 1975). Correlations of $\dot{V}O_2$ and blood flow at 15 and 60 min sample intervals were found to be 0.76 ($p < 0.05$) and 0.75 ($p < 0.01$), respectively (Appendix X, Tables 3 and 4).

Venous blood oxygen tensions during work in this study (Appendix VII, Table 4) did not approach muscle $\dot{V}O_2$ limiting values of 13 mmHg as reported by Stainsby et al. (1972) and Stainsby (1973). Wenger and Reed (1976) have stated that it is possible since venous blood represents total muscle drainage and not actual drainage of only those fibers which are extracting oxygen, that inflated P_VO_2 data may be inaccurately assessed. This could not be the case in the present experiments since all fibers were recruited by the applied electrical stimulus (Appendix VIII) (Kugelberg and Edström, 1968) and the dog gastrocnemius is homogeneous with respect to highly oxidative fiber types (Appendix VIII).

In summary, although some alterations in selected parameters were noted in present experiments, good viability was maintained.

ELECTRICAL STIMULATION AND MUSCLE METABOLISM. The decision to employ direct muscle stimulation was a result of preliminary work. Although Hirche et al. (1971) have reported no differences as to metabolic effects associated with nerve versus direct muscle stimulation, pilot work in the present study led to doubts of nerve viability during prolonged electrical stimulation of this tissue.

The choice of rhythmic tetanic trains as the temporal pattern of muscle stimulation was based on reports which suggested that in vivo

muscle contraction is predominantly tetanic, as opposed to twitch, in nature (Bigland and Lippold, 1954; Barclay and Stainsby, 1972). Further, it was known that metabolic requirements of the muscle could be successfully varied by altering the intratrain stimulation rate (DiPrampero et al., 1969; Horstman, et al., 1976). Preliminary study to establish the temporal variations in pattern of stimulation required to produce slightly elevated and highest $\dot{V}O_2$ and blood flow for any overall stimulation rates resulted in the selection of the patterns of stimulation reported here.

MUSCLE METABOLIC RATES DURING CONTRACTION. Resting values for blood flow, oxygen uptake and lactate output (Tables 3 and 4) were consistent with previous reports of the in situ dog gastrocnemius muscle preparation (Chapler and Stainsby, 1968; DiPrampero et al., 1969; Hirche et al., 1970b). Therefore the fact that both mild and severe stimulation patterns in this study produced metabolic responses below maximum reported patterns provided initial indications that contractions were primarily aerobic in nature.

Blood flow responses to stimulation reached maximum mean values of approximately 60 ml/min in contrast to reports where blood flow in similar models exceeded 120 ml/min (Hirche et al., 1970b, 1975).

Greatest oxygen uptake responses in the present studies were similarly low, 8 fold over resting values, compared to accepted maximum $\dot{V}O_2$ for this muscle preparation in the order of 20 to 40 fold above resting metabolism (Stainsby and Welch, 1966; Chapler and Stainsby, 1968; Hirche et al., 1970b, 1975; Horstman et al., 1976).

Blood flow restrictions of oxygen uptake increases were not obvious as evidenced by the mean P_AO_2 and $P_{\bar{V}}O_2$ values discussed earlier. Moreover, Hirche et al. (1970a) claim that flow is facilitated by rhythmic compression of veins during contraction in the isolated in situ muscle

preparation. Rhythmic patterns of muscular contraction are known to produce a similar in vivo pattern (Hudlicka, 1973).

Maximum observed lactate outputs by the present muscle preparation also supported the suggestion of oxidative metabolism during the selected sample intervals (Table 4). Highest reported lactate outputs by similar muscle preparations under chloralose-urethane anesthesia (Hirche et al., 1970b, 1975) and barbituate anesthesia (Stainsby and Welch, 1966; Chapler and Stainsby, 1968) were approximately 2 and 4 fold, respectively, above those reported here. Comparison of present lactate output values with those reported by Stainsby and coworkers must be considered cautiously because of their use of barbituate anesthesia (Dunn and Critz, 1975).

Although present evidence of unlimited oxygen supply to the muscle is compelling, further considerations were important. Barclay and Stainsby (1975) have reported that maximum metabolic rate cannot occur in this muscle preparation at blood pressures associated with barbituate anesthetized dogs. The factors responsible for flow dependent $\dot{V}O_2$ were not established but evidence indicated that it was not oxygen limited. Halothane, a hypotensive drug, reduced perfusion pressure in present experiments and may therefore have limited muscle blood flow (Hudlicka, 1973). Experiments employing rhythmic twitch patterns of stimulation showed marked differences in $\dot{V}O_2$ between frequencies of 1 and 5 twitches per sec (Chapler and Stainsby, 1968; Barclay and Stainsby, 1972). Further, rhythmic tetanic stimulation, derived from varied trains of impulses delivered to dog gastrocnemius muscle, have also produced large differences in $\dot{V}O_2$ under mild versus severe stimulation conditions. DiPrampero et al. (1969) have varied impulse trains between 0.2 sec on to 2.8 sec off and 1.2 sec on to 1.8 sec off. Oxygen uptake increases 10 fold over rest with the severe stimulus and a 2 fold difference was noted between mild and severe stimulation patterns

for $\dot{V}O_2$. Horstman et al. (1976) have conducted studies of dog gastrocnemius muscle employing variations in the numbers of impulses per train while keeping the intratrain stimulation frequency constant at 40 stimuli per sec. Marked differences in $\dot{V}O_2$ between stimulus severities were noted over a continuum from near resting $\dot{V}O_2$ up to accepted maximum $\dot{V}O_2$ values of 107 µl/g/min. Trains of impulses in this study of 0.08 sec on to 2.7 sec off and 1.4 sec on to 2.7 sec off resulted in far less variation in $\dot{V}O_2$ between the mild and severe intensity groups than was anticipated. Failure to observe expected metabolic differences between mildly and severely stimulated muscle in present studies might be attributed to flow limited $\dot{V}O_2$ in the severe group, but this effect was not mediated by hypoxia.

An alternative suggestion is that maximum $\dot{V}O_2$ of the in situ muscle could not be established by trains of impulses of the pattern employed here. Fales et al. (1960) and Stainsby and Fales (1973) have reported that $\dot{V}O_2$ per stimulus is lower when large groups of stimuli are delivered continually to a muscle. In effect, this could indicate energy conservation as a result of fusion of responses to each impulse (Fales et al., 1960). Certainly the methods and results of DiPrampero et al. (1969) are closer to present efforts than are those reports employing twitch stimulation. However, Hirche et al. (1970b, 1975), while employing trains of impulses (0.2 sec on to 0.5 sec off), have repeatedly found muscle $\dot{V}O_2$ in excess of 120 µl/g/min, the accepted maximum for the in situ muscle preparation during both rhythmic twitch and tetanic stimulation.

The findings of Horstman et al.(1976) further complicate this issue in that although large differences in $\dot{V}O_2$ were noted for variations in

impulse trains, these authors were opposed to the suggestion of Barclay and Stainsby (1975). Horstman and coworkers concluded that maximum $\dot{V}O_2$ in their muscle preparation was normally limited by oxygen delivery rather than intrinsic muscle limitations.

In summary, if oxygen is not the mediator of flow dependent maximum muscle $\dot{V}O_2$ (Barclay and Stainsby, 1975), the less than expected differences in metabolic rates between mild and severe stimulation patterns in the present study may reflect the stimulus pattern employed here or may be attributable to yet unestablished factors in blood or muscle.

ARTERIAL-VENOUS DYNAMICS. The use of the Schlein correction (Schlein et al., 1973) for water loss from plasma during transit through skeletal muscle limited direct comparison of absolute values of present arterial-venous differences with previous publications. In addition, reports of pyruvate and amino acid arterial-venous differences in this study (Tables 4 and 5) constitute initial records of these events from the in situ dog gastrocnemius muscle preparation. Nonetheless, trends in other blood-borne metabolites in this study should be comparable with data reported elsewhere, provided that anesthesia effects are not confounding.

The similarities between present lactate output and previous reports of the parameter have been discussed.

The small glucose uptake witnessed here in resting in situ muscle (Table 4) has been documented (Bass and Hudlicka, 1960; Chapler and Stainsby, 1968; Corsi et al., 1969; DiPrampero et al., 1969; Hirche et al., 1970b). Although the alteration of glucose uptake that accompanied both intensities of electrical stimulation in this study (Table 4) conform to reported trends (Chapler and Stainsby, 1968; DiPrampero et al., 1969; Hirche et al., 1970b), the severe stimulus employed here produced glucose

uptakes which were consistent with submaximal exercise levels (Chapler and Stainsby, 1968; Hirche et al., 1970b).

The slight, consistent output of free fatty acids by the resting muscle preparation (Table 4) under a variety of anesthetic agents has also been reported earlier (DiPrampero et al., 1969; Hirche et al., 1970b). This is in contrast to in vivo occurrences marked by a small resting uptake of fatty acids by dog skeletal muscle (Paul and Issekutz, 1967; Paul, 1970). The mediating factors and metabolic significance of fatty acid release from resting in situ muscle preparation is unclear at present. However, it could be conjectured that anesthesic agents in general produce imbalances in intramuscular fat metabolism at the level of lipolysis, re-esterification or β-oxidation.

With prolonged contractile activity, substantial increases in free fatty acid uptake occur in dog skeletal muscle preparations (DiPrampero et al., 1969; Hirche et al., 1970b). The extent of free fatty acid uptake demonstrated by Hirche et al. (1970b) increased during the initial adaptive period in tension development (0 to 13 min) and thereafter remained relatively constant. Although present data (Table 4) was more variable than that reported by Hirche et al. (1970b), free fatty acid uptake approximated the trends observed earlier (DiPrampero et al., 1969; Hirche et al., 1970b). The magnitude of the present trends were consistent with the two distinct aerobic demands.

The very slight output of pyruvate from resting or rhythmically contracting dog gastrocnemius muscle has not been previously documented. However, outputs of this magnitude were expected since very low concentrations of pyruvate have been found in the venous drainage of dog limbs (Secord and Russell, 1973; Tipton et al., 1974).

MUSCLE TISSUE CONCENTRATIONS OF GLYCOGEN AND TRIGLYCERIDES. Note: Muscle concentrations of the selected metabolites in this study were examined by comparison of left stimulated muscle with right non-stimulated muscle (Table 7) [i.e., tissue samples for both stimulated and non-stimulated muscle were collected at the completion of the stimulation period (Figure 3)]. This scheme provided an index of experimental viability in that muscle metabolite concentration changes in non-stimulated muscle of the four intensity-duration combinations should reflect any deterioration of the muscle preparation with time. Of the muscle metabolites assayed, only triglyceride concentration demonstrated a consistent trend in this regard (Table 7). Comparison of right non-stimulated muscle in the four groups revealed a trend, though not significant ($p > 0.05$), for lower triglyceride concentrations in the M65 and S65 groups when compared to the M20 and S20 groups. The significance of this is unclear, but did not appear to indicate loss of viability with time since other muscle metabolites did not demonstrate a similar trend in these same groups (Table 7).

Resting muscle glycogen concentrations in this study (Table 7) were low compared to earlier reports of dog gastrocnemius muscle (Chapler and Stainsby, 1968; Corsi et al., 1969; Chapler and Moore, 1972) and inter-group concentration differences were noted at rest (M65 > M20, S20, S65; Table 7). Although the suggestion that initial glycogen concentrations in muscle may limit the capacity of a muscle for prolonged intense effort (Saltin and Karlsson, 1971a; Hultman and Bergström, 1973), it did not appear to affect tension development in this study (Figure 4). However, the effects of initial differences of muscle glycogen content on exercise glycogen depletion patterns and related metabolic events are noteworthy.

Glycogen depletion rates in the M20 and S20 groups, which showed similar initial glycogen content, were approximately 2 fold different in terms of both relative rates (left to right ratios) and absolute rates (Table 7). Glycogen disappeared at 0.17 and 0.38 μmoles of glucosyl units/g/min over 20 min of mild and severe exercise, respectively. These changes were comparable to 17 and 42% decreases in initial concentrations based on left to right ratios. On the other hand, glycogen content at rest in the prolonged groups were 2 fold greater in the M65 than S65 group (Table 7). After 65 min of stimulation, approximately 25 and 61% of the initial glycogen content of mild and severely stimulated muscle had been depleted. Yet, glycogen had been catabolized at equal absolute rates from these initial pools, 0.065 μmoles of glucosyl units/g/min in both intensities of exercise.

The fact that initial glycogen content influences rate of breakdown has been reported earlier (Klausen et al., 1975; Essén et al., 1975). However, variability in present glucose and lactate arterial-venous differences (Table 3) did not support or refute the suggestion that as work progresses and glycogen is depleted, blood glucose (Wahren et al., 1975) and lactate (Essén et al., 1975) become increasingly important to the maintenance of a required muscle carbohydrate supply. This effect, if apparent, would have been noted in comparisons of the M20 and M65 groups where pre-exercise glycogen concentrations were 1.75 fold greater in the M65 group (Table 7) and intensity of work was equalized by electrical stimulation. On one hand, present glucose arterial-venous difference suggests a trend toward a lower glucose uptake in the M65 group at 15 min compared to the M20 group at this same period (Table 3). But, trends in lactate arterial-venous differences also indicate greater lactate production in the M20 than M65 group at 15 min (Table 3).

The large variability of muscle triglycerides (Table 7) resulted in no relationships between muscle at rest or following exercise. Previous observations in long term, maximally contracting dog *in situ* gastrocnemius muscle preparations, suggested that triglycerides in muscle are used when the supply of plasma free fatty acids is lower than the demand for β-oxidation in muscle (DiPrampero *et al.*, 1969; Barclay and Stainsby, 1972). Present data (Table 7) do not compare consistently with reports of 13.6 ± 0.9 μmoles of triglyceride/g/wet weight in resting dog gastrocnemius muscle (Barclay and Stainsby, 1972). The differences between present and earlier observations most likely reflect technical problems inherent in the triglyceride assay. Large variations (20%) in left to right muscle concentrations under resting conditions have been noted for small sample sizes from dog gastrocnemius muscle (Barclay and Stainsy, 1972) and for total muscle homogenates of monkey muscle (Masoro *et al.*, 1964). The technical difficulties of cleaning muscle samples of intercellular fat depots has been proposed as a major limitation to direct study of intramuscular triglycerides (Paul, 1975). An alternative suggestion to that discussed above might involve triglyceride concentration changes in right non-stimulated muscle as a result of the experimental protocol (Figure 3).

Resting pre-stimulation muscle preparations demonstrated small consistent free fatty acid outputs (Table 4). This effect could account for the decreasing triglyceride concentrations in the right non-stimulated muscle of the 65 min groups since this muscle was not examined until the completion of each experimental period. Moreover, the effects of stimulation of the left muscle and its communication with the total dog for the experimental period could act through some unestablished series of events to alter fat metabolism in the right non-stimulated muscle. A

further suggestion, previously mentioned (pp. 76) is the possible effects of anesthetic agents on intramuscular fat metabolism.

BALANCE OF SUBSTRATE UTILISATION FOR OXIDATIVE METABOLISM. Estimation of the percentage of muscle $\dot{V}O_2$ accounted for by carbohydrate oxidation ($\%\dot{V}O_2(CHO)$) (on the basis of net exchange of metabolites with blood perfusing the muscle) revealed that carbohydrate oxidation accounted for only 10 to 14% of the resting $\dot{V}O_2$ (Table 8). This is in keeping with prior reports of the preponderance of fat oxidation by resting dog muscle, both *in situ* (Chapler and Stainsby, 1968; DiPramperq *et al.*, 1969; Hirche and Vollmer, 1970; Barclay and Stainsby, 1972) and *in vivo* (Paul and Issekutz, 1967; Issekutz and Paul, 1968; Paul, 1970).

Estimates of $\%\dot{V}O_2(CHO)$ for exercise conditions revealed trends which were generally consistent with previous reports of the major role played by carbohydrates in support of exercise muscle metabolism, *in situ* (Chapler and Stainsby, 1968; DiPrampero *et al.*, 1969; Barclay and Stainsby, 1972). The fact that severe stimulation required 62 and 44 $\%\dot{V}O_2(CHO)$ at 15 and 60 min, respectively as compared to mild stimulation requiring 76 and 58 $\%\dot{V}O_2(CHO)$ at these same respective intervals suggested that the two exercise intensities were not sufficiently different for variations in $\%\dot{V}O_2(CHO)$ to occur. Certainly, 62 versus 76 and 44 versus 58 $\%\dot{V}O_2(CHO)$ values are within accepted limits of the errors inherent in the assumption underlying $\%\dot{V}O_2(CHO)$ estimates.

A comparison of these estimates with data from DiPrampero *et al.* (1969) support this conjecture. By subjecting similar preparations to mild and severe stimuli, they showed muscle $\dot{V}O_2$ differing by 2 fold (45.6 to 88.5 ml/kg/min) compared to 1.8 fold differences in $\dot{V}O_2$ in the present

intensity groups (Table 3) (48.6 to 86.2 µl/g/min). Yet, mean RQ values related to their observed $\dot{V}O_2$ were 0.86 ± 0.05 and 0.89 ± 0.03, respectively, which suggested similar large contributions of carbohydrates to oxidative processes in both intensities of prolonged exercise (3 hr). Their estimates of $\%\dot{V}O_2(CHO)$, based on muscle-blood dynamics of metabolites, also supported similarities in the preponderance of carbohydrate oxidation in the two intensity groups. However, both present data and that of DiPrampero et al. (1969) are in contrast to that of Chapler and Stainsby (1968) who noted very large differences in exercise intensity effects on estimates of $\%\dot{V}O_2(CHO)$. Their work on prolonged exercise, however, must be considered with caution in that the use of barbituate anesthesia may have inhibited fatty acid oxidation (Dunn and Critz, 1975) in the in situ gastrocnemius muscle preparation.

THE PATTERN OF MUSCLE AMINO ACID METABOLISM. Since the inferences which can be drawn from present data attends the reliability of quantitative analysis of amino acids, it is essential that evidence be provided in this vane. As stated earlier, direct support for the extent of muscle-blood amino acid dynamics observed in this study (Table 5) are unavailable since present data constitute initial reports of these events in the dog gastrocnemius muscle preparation. However, indirect evidence of the reliability of present measurements was found.

Weissel et al. (1973) have reported resting tissue concentrations of free glutamate and aspartate from dog "calf" muscles. Intramuscular free contents of these amino acids were 3.60 ± 0.14 and 0.30 ± 0.02 µ moles/g/ wet weight for glutamate and aspartate, respectively, compared to 3.99 ± 0.44 and 0.41 ± 0.04 µmoles/g wet weight for these same respective metabolites in this study (Table 7). In addition, amino acid

concentrations of femoral arterial blood from anesthetized dogs in this study (Appendix VII, Table 2) compare very favorably with reports of these parameters in intact resting dogs (Hier and Bergeim, 1946). Examination of resting arterial concentrations of the selected amino acids and the free pools of these same amino acids in resting skeletal muscle in this study demonstrated proportional concentrations (Table 7 and Appendix VII, Table 2). Therefore, the report that resting arterial blood concentration of any one amino acid is reflected in the relative size of the muscle free pool of that amino acid (Baños et al., 1973) is supported by the present data.

The observed increase in the muscle free pool of aspartate and the decrease in the muscle free pool of glutamate (Table 7) were expected in exercise (Borst, 1962; Edington et al., 1973; Koziol and Edington, 1975). But alanine was found in unchanged or diminished intramuscular free concentrations dependent upon the intensity-duration combinations of muscular activity (Table 7). As judged from left to right ratios the alanine content in muscle decreased 31 and 45% in mildly exercised muscle at the 20 and 65 min sample intervals, respectively. Severe stimulation produced 5 and 34% decrements in the muscle free alanine pool at these same respective intervals. This is contrasted to reports of unchanged or increased intramuscular contents of free alanine during 10 min of electrical stimulation of in vitro skeletal muscle of rats (Edington, 1973; Koziol and Edington, 1975). Reports of muscle alanine dynamics in preparations similar to the present muscle model, which have been stimulated for periods of time up to 1 hour, are not available at this time.

Ahlborg et al. (1974) witnessed progressive increases in output of alanine with time up to 4 hr from the leg muscles of exercising men (work

load equal to 30% of $\dot{V}O_2$ maximum). Further, Felig and Wahren (1974) have reported that alanine production in skeletal muscle of exercising men responds directly to exercise intensity. Alanine outputs during mild moderate and heavy exercise were 1.5, 1.9 and 5 fold above resting alanine output, respectively. While exercise alanine output increases reflected the intensity of exercise in present experiments (Table 5), a decline in alanine output was noted between 15 and 60 min in both exercise intensities.

The discrepancies between present observations of alanine dynamics and earlier reports noted above, must certainly reflect differences in the experimental models. Volitional exercise in man involved recruitment patterns quite different to the present (Gollnick et al., 1973d, 1974a). The fact that $\dot{V}O_2$ in the exercising subjects of Ahlborg et al. (1974) was at an apparent steady state level for 4 hr, is definitely contrasted to exercise metabolic events in electrically stimulated in situ muscle. Electrical recruitment of all fibers in the dog gastrocnemius muscle appeared to result in decreasing $\dot{V}O_2$ in the muscle between 15 and 60 min (Table 3) although tension declines were not obvious in this same period (Figure 4). Similar phenomena have been reported for the in situ muscle model (Chapler and Stainsby, 1968; Hirche et al., 1970b), but the basis of this effect is as yet unexplained. Fatigue and lack of contribution to tension development or metabolism in some muscle fibers might be expected as stimulation is prolonged. However, $\dot{V}O_2$ and tension changes are not parallel, suggesting that these events are mutually exclusive. The parallel between alanine arterial-venous difference changes (Table 5) and other metabolic changes (Tables 3 and 4), therefore suggests decreasing metabolic rate in the muscle preparation, even though an appar-

ent steady state of tension development was occurring (Figure 4).

The unanticipated changes in intramuscular free alanine may in part reflect the above considerations. But other events may also be involved. The tendency, though not significant ($p > 0.05$), for arterial concentrations of the selected free amino acids to decrease during the experimental periods may be implicated. It is known that muscle tissue concentrations of amino acids are regulated closely with the concentrations of amino acids in the blood stream (Baños et al., 1973). It could also be proposed that increased intramuscular metabolite concentrations (i.e., lactate) associated with muscular exercise (Hirche et al., 1971) could affect water accumulation in the muscle (Table 4) and muscle water uptake could dilute existing pools. Increased metabolite concentrations in muscle on the other hand might also provide the impetus for passive loss of amino acids as a result of an altered osmotic balance. However, the observation that glutamate and aspartate contents of muscle (Table 7) behaved as previously reported does not support any of the above conjectures. This may mean that observations of alanine were not biased by external forces or that alanine loss from muscle is mediated differently than glutamate or aspartate loss. The latter is opposed by reports of Baños et al. (1973).

In summary, the cause or causes of progressive alanine loss from muscle during exercise in the present studies remains obscure, but probably reflects the events discussed above. This effect could prove beneficial to enhanced metabolism. Glutamate-pyruvate transaminase in skeletal muscle is characterized by a Michaelis constant near equilibrium for the concentration ranges of both substrates and products in resting muscle (Young, 1970). The fact that glutamate concentrations decreased

in muscle during exercise (Table 7), while pyruvate increased (Table 4), might perturb the balance of glutamate-pyruvate transamination reciprocally (i.e., no net effect on transaminase activation), if the end products of the pathway, alanine and αKG, are not removed. Therefore, alanine decreases in muscle may reflect a normal physiological reaction associated with enhancement of the rate of alanine production from glutamate-pyruvate transaminase.

THE IMPORTANCE OF GLUTAMATE-PYRUVATE TRANSAMINATION. The evidence presented in this study may be taken to support the view that coupled transaminations involving glutamate-pyruvate transamination, supply net CAC intermediates in skeletal muscle during 2 steady state aerobic exercise intensities. Mean values for muscle glutamate levels fell while muscle aspartate contents rose (Table 7) as might be expected in meeting the two respiratory demands (Borst, 1962; Koziol and Edington, 1975). However, it should be noted that this observation is contrasted to events in cardiac muscle where aspartate concentrations decrease in conjunction with glutamate and alanine production (LaNoue et al., 1970; Randle et al., 1970; Safer and Williamson, 1973). These latter observations in heart muscle were the basis for a proposed coupling of glutamate-pyruvate transamination with glutamate-oxaloacetate transamination for CAC repletion (Safer and Williamson, 1973). The contrast in aspartate and glutamate dynamics between cardiac and skeletal muscle therefore suggests that the proposal of Safer and Williamson (1973) for CAC repletion does not apply in skeletal muscle. The alternative suggestion by Davis et al. (1972) however, appears valid. They suggested coupling of branched chain amino acid transaminases with glutamate-pyruvate transaminase. Skeletal muscle is known to oxidize isoleucine, leucine and valine in large amounts (Odessey and

Goldberg, 1972; Goldberg and Odessey, 1972; Beatty et al., 1974; Odessey et al., 1974). Odessey et al. (1974) have indirectly demonstrated the coupling of the reactions proposed by Davis et al. (1972) when they revealed that alanine output by rat diaphragm muscle may account for all amino groups removed from catabolized branch chain amino acids.

Although not definitive, correlations in this study involving muscle glutamate concentration support the above interpretations. Tissue glutamate correlated negatively with muscle oxygen uptake at 15 min ($r = -0.58$) and 60 min ($r = -0.63$). Alanine dynamics further substantiate the occurrence of transamination events. Estimated de novo alanine output by muscle was 1.8 fold greater in the severe group than mild treatment group at 15 min, though no difference occurred between intensity groups at 60 min. Tissue alanine concentration varied directly with $\dot{V}O_2$ ($r = 0.56$) and inversely with tissue glutamate concentration ($r = -0.65$) at 15 min.

Molé et al. (1973) have shown that glutamate-pyruvate transaminase occurs in distinct cytoplasmic and mitochondrial forms. It is also accepted that the CAC is regulated at αKG dehydrogenase and citrate synthase such that αKG or OAA can be partitioned into the malate-aspartate shuttle or proceed through the CAC (Figure 1) (LaNoue and Williamson, 1971; Safer, 1975). These regulations provide for transient disruption of uniform flux in the CAC such that balanced glycolytic and CAC fluxes allow more efficient oxidative metabolism of carbohydrates and other fuels under a wide variety of metabolic conditions.

The question may therefore be asked that, if coupled transamination does occur in skeletal muscle, in which compartment is that occurrence of greatest metabolic significance? If the cytoplasmic event predominates, less drain on the CAC intermediate pool is required in that coupled trans-

amination may offer an alternative to lactate production for reoxidation of cytoplasmic NADH (Felig and Wahren, 1971a). If mitochondrial transaminations predominate, net CAC supplementation results.

Correlations of tissue glutamate and tissue alanine with pyruvate and lactate arterial-venous differences at 15 min do not suggest a preponderance of coupled transamination events in the cytoplasm. Tissue glutamate correlated directly with pyruvate ($r = 0.68$) and lactate ($r = 0.83$) arterial-venous differences while tissue alanine correlated inversely with pyruvate ($r = -0.56$) and lactate ($r = -0.59$) arterial-venous differences. While cause and effect cannot be deduced from a correlation, present results and reports by Molé et al. (1975) do suggest that where pyruvate and lactate production in exercising muscle was greatest, tissue glutamate was least depleted and alanine was produced less quickly (i.e., little glutamate-pyruvate transamination activity).

It was proposed that glutamate-pyruvate transamination acting through coupled transamination in the cytoplasm was an alternative pathway to lactate production in the oxidation of NADH (i.e., direct repletion of malate-aspartate shuttle intermediates in the cytoplasm). It appears that this was not the case at 15 min in this study. Where greater amounts of pyruvate were produced and not oxidized in the CAC, pyruvate may have competed very favorably for NADH with the malate-aspartate shuttle to produce lactate. Either the malate-aspartate shuttle was operating at its full capability or there was a need for more rapid reoxidation of NADH than the shuttle could provide. It is known that pyruvate reduction to lactate is capable of very rapid reoxidation of NADH (Lehninger, 1970). The evidence therefore allows conjecture that at 15 min, mitochondrial support of the CAC was the more important aspect of coupled transamination

since relationships in present data do suggest that these events were occurring (pp. 85). Similar evidence for occurrence of compartmentalized glutamate-pyruvate transamination at 60 min was not indicated from available data.

Estimation of αKG production from glutamate-pyruvate transamination (Table 9) and comparison of the magnitude of this occurrence with the malate-aspartate shuttle, CAC, and total muscle needs for OAA flux (Figures 5, 6 and 7) has demonstrated that at each intensity of exercise, de novo αKG from coupled transaminations, supplied similar proportions of the OAA needs of the tissue. α-Ketoglutarate from glutamate-pyruvate transamination was found to account for 16 to 22% of CAC flux or 19 to 30% of malate-aspartate shuttle flux in mild stimulation while in severe exercise, 10 to 17% of the CAC flux or 22 to 23% of the malate-aspartate shuttle flux was accounted for by this same source (Figure 6). These ratios represent total diversion of αKG from coupled transaminations to either the CAC or malate-aspartate shuttle. A composite comparison (Figure 7) showed 9 to 12% and 7 to 10% of total tissue needs for OAA flux could be accounted for by de novo αKG generation via coupled transaminations at 15 and 60 min, respectively.

Odessey et al. (1974) have estimated the potential role for energy production in human skeletal muscle of amino acids. Based on data for alanine release, ATP production and oxygen consumption of human muscle, these authors have suggested that coupled transaminations of glutamate-pyruvate transaminase and branched chain amino transaminases may account for 14% of the total oxidative energy supply. This figure is quantitatively similar to current findings outlined above.

While concentration changes may not be held as indicators of flux,

it is clear in this investigation that de novo generation of αKG from glutamate-pyruvate transamination does in fact reflect the magnitude of concentration change in the CAC which is traceable to this pathway. On a subjective basis, the rates of input of de novo αKG (Table 9) to the CAC in the present muscle model appear small for the submaximal exercise intensities studied here and at the time intervals considered. It might be conjectured that these apparently small concentration changes have little effect on CAC repletion. This of course must be considered cautiously since major changes in the CAC intermediate pool must likely occur in the early minutes of exercise adaptation. However, subsequent small input of αKG from glutamate-pyruvate transamination during exercise may well have major importance in sustaining CAC concentrations in the range of sensitive Michaelis constants of most CAC enzymes (Atkinson, 1969; Garland et al., 1969; Tager et al., 1969; Utter et al., 1969).

The following suggestions for future research therefore seems appropriate. Major alterations in the CAC may occur during the initial minutes of exercise adaptation. This time interval was not investigated in the present study since primary interest was focused on sustained metabolic patterns, however, research focused on CAC repletive mechanisms in the initial exercise period in homogeneous oxidative muscle is indicated. Such research must investigate the balance of changes in all CAC intermediates as well as the amino acids glutamate, aspartate, alanine, leucine, valine and isoleucine. In addition, research is also indicated as to the Michaelis constants of CAC enzymes in skeletal muscle and their sensitivity to net changes in the CAC intermediate pool from coupled transaminations over a variety of sample intervals and for a full spectrum of exercise intensities.

IMPLICATIONS FOR EXERCISING MAN. It has been stated that it is not clear

why the modest carbohydrate stores in muscle and liver play a major role in prolonged aerobic exercise, particularly since depletion of these stores places a limitation on continued activity. Not only does fat constitute a far greater energy reserve (i.e., 10 kg of triglyceride in adipose tissue compared to 400 g of carbohydrate in liver and muscle in a 70 kg man), but on a weight relative basis, triglycerides supply by oxidation about 2.5 fold more ATP than carbohydrate (Bartley et al., 1970). Furthermore, present data (Tables 4, 5 and 8) and earlier reports (Keul et al., 1972) suggest that while carbohydrate was supplying a large proportion of the oxidized substrate to muscle, a large portion of incompletely metabolized carbohydrate was also ultimately lost from the muscle as lactate, pyruvate and alanine.

High rates of fatty acid oxidation which also accompany prolonged aerobic activity (Saltin, 1973) appear to inhibit pyruvate oxidation (Newsholme and Start, 1973). Since glycogenolysis is not geared to the rate of pyruvate oxidation but to relative work intensity, "excess" pyruvate is produced beyond either the need or capacity of the tissue to oxidize this substrate (Molé et al., 1973). The suggestion that lactate, pyruvate and alanine output from muscle provides gluconeogenic substrates (Felig and Wahren, 1971b) does not alter the evidence supporting apparently inefficient use of limited carbohydrate stores. Not only does the rate of splanchnic gluconeogenesis lag behind the rate of liver glycogen depletion but lactate, pyruvate and alanine cannot provide substrate for a net effect of gluconeogenesis in the organism since these substrates were originally derived from carbohydrate precursors.

Based on the accumulated evidence above, Saltin (1973) has therefore suggested that man is not metabolically designed or adapted for the

demands imposed by prolonged exercise. In light of the non-physiological innervation and the use of a muscle model with only high oxidative capacity, present research findings can neither refute nor support this statement. However, the data are sufficiently suggestive to warrant consideration of the following possibilities. The fact that "excess" pyruvate from glycolysis may play a small role in repletion of the CAC in electrically stimulated *in situ* dog gastrocnemius muscle suggests inefficient use of limited carbohydrate stores during prolonged submaximal exercise. The finding that amino acids account for 7-12% of oxidative metabolism on one hand, suggests a sparing of other substrates. However, catabolism of leucine, isoleucine and valine appears to require coupled transaminations with glutamate-pyruvate transaminase (Odessey *et al.*, 1974). In other words, amino acid oxidation does not spare carbohydrates; pyruvate is simply derived from oxidative processes and lost as alanine output. The implications of such events for the *in vivo* musculature of exercising man are clear, however, further research in this vane is required in order to clarify the physiological significance of such events in the *in vivo* human muscle.

SUMMARY

1) Application of "mild" and "severe" trains of electrical impulses to the isolated, in situ dog gastrocnemius produced two submaximal intensities of contractile activity with respect to metabolic rate.

2) In the present muscle model, under the submaximal metabolic conditions noted above, and at the time intervals examined, αKG from coupled transaminations involving glutamate-pyruvate transaminase, was estimated to provide (a) similar proportions (7-12%) of the OAA flux of the stimulated muscles during two intensities of steady-state submaximal exercise, and (b) a potentially small concentration change in the CAC intermediate pool itself. The latter observation may suggest the following alternatives which provide a basis for future research.

    1. large changes in the CAC intermediate concentrations may not occur in skeletal muscle during exercise of the intensity reported here.
    2. if large changes in the CAC intermediate pool did occur these may not be accompanied by a large alanine production.
    3. large changes in the CAC intermediate pool may have occurred prior to the sample intervals reported here.
    4. large changes in the CAC intermediate pool may not occur at any time during any exercise intensity. In effect, small repletive effects of the magnitude noted here may have major influences on the sensitive Michaelis constants of most CAC enzymes.

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APPENDIX I

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

A large body of evidence now exists in support of the citric acid cycle as the major, if not sole pathway in skeletal muscle for oxidation of acetic acid residues (from carbohydrates, fatty acids and ketone bodies) and of other substrates (amino acids) capable of being converted to intermediates of this cycle. But, the functional complexity of the cycle, established over several decades of research, suggests that our understanding of the basic processes is far from complete. The intracellular compartmentation of the enzymes and substrates of the cycle, specific functions of intra- and extramitochondrial portions of the cycle and the specific functions of multiple alternative reactions related to the cycle intermediates all serve to emphasize the diversity of current problems (Lowenstein, 1969; Mehlman and Hanson, 1972).

The dependence of many biochemical functions in skeletal muscle on a few reactions of the CAC has great implication for metabolic regulation of enzyme kinetics and concentrations of CAC intermediates. Where a metabolite serves as a substrate for two or more enzymes, the substrate concentration and relative affinities of the enzymes for the common substrate are major factors in determining the fraction of the intermediate that interacts with each. Neither concentration nor enzyme affinity need be fixed. By modulating concentrations and enzyme activities, the proportion of a branch point metabolite which is metabolized through each of the alternative pathways can be caused to vary appropriately with changing metabolic needs (Newsholme and Start, 1973).

Metabolic conditions appear to dictate the total amount of CAC intermediates present in mammalian cardiac muscle (Bowman 1966; Penney and Cascarano, 1970; Neely et al., 1972; VonKorff, 1972; Davis and Bremer, 1973; LaNoue et al., 1973; Safer, 1975), avian skeletal muscle (Krebs and Eggleston, 1940; Borst, 1962), insect skeletal muscle (Sackton and Wormser-Shavit, 1966; Johnson and Hansford, 1975) and mammalian skeletal muscle (Lowensten, 1972a, b; Koziol and Edington, 1975). The fact that these changes in CAC intermediate pools do occur and are not merely a re-shuffling of CAC contents already present, implies the presence of mechanisms which make possible a net increase or decrease in the contents of the citric acid cycle.

REPLETION OF CITRIC ACID CYCLE INTERMEDIATES. For optimal function in the case of increased respiratory demand, the CAC requires both two carbon residues from acetyl-CoA and OAA (or any of the dicarboxylic acids which may be metabolized to OAA such as malate, succinate, fumarate or αKG). However, an increased rate of acetyl-CoA entry into the CAC cannot result in net increases in the total CAC intermediate pool. The condensation of acetyl-CoA with available OAA may provide for transient realignments of a portion of the CAC intermediates due to re-shuffling of available pools and although OAA is regenerated as an overall result of the cycle, the emergent carbon structure is not that which entered the cycle as citrate. The resultant OAA contains only two carbons of the original OAA and the other two are derived from acetyl-CoA. Two molecules of the original OAA are lost as CO_2 per cycle turnover and net additional OAA is not produced (Lehninger, 1970).

Changes in the total cycle intermediate pool therefore requires precursors which are themselves not originally part of the CAC. Mechan-

isms which may serve this role in a variety of tissues fall into two general categories, carboxylation of di- and tricarbon structures to CAC dicarboxylic acids and metabolism of amino acids to CAC intermediates.

CARBOXYLATION OF DI- AND TRICARBON SUBSTRATES TO CITRIC ACID CYCLE DICARBOXYLIC ACIDS IN MUSCLE TISSUES. In animal tissues, there are four known enzymatic reactions which do form CAC intermediates from 3 carbon precursors. These pathways involve the enzymes, pyruvate carboxylase (Utter and Keech, 1963) (Equation 1), propionyl-CoA carboxylase (Tietz and Ochoa, 1959) (Equations 2 and3), phosphoenolpyruvate carboxykinase (Utter and Kurahashi, 1954) (Equation 4) and malic enzyme (Ochoa et al., 1948) (Equation 5).

(1) pyruvate + CO_2 + ATP $\rightleftharpoons$ oxaloacetate + ADP + P_i

(2) propionyl-CoA + CO_2 + ATP $\rightleftharpoons$ methylmalonyl-CoA + ADP + P_i

(3) methylmalonyl-CoA $\rightleftharpoons$ succinyl-CoA

(4) phosphoenolpyruvate + CO_2 + GDP $\rightleftharpoons$ oxaloacetate + GTP

(5) pyruvate + CO_2 + TPNH $\rightleftharpoons$ malate + TPN

Present evidence suggest that although pyruvate carboxylase plays a major role in liver, kidney and adipose tissue metabolism (Scrutton and Utter, 1968), it is generally conceded that this enzyme is very low in activity or absent from mammalian muscle tissues (Scrutton and Utter, 1968; Lehninger, 1970; Mayes, 1971). This has been supported by Ballard et al. (1970) who have found the presence of pyruvate carboxylase in rat muscle. However, the activity of the enzyme in rat muscle was only 1% of that found in rat liver, kidney and adipose tissue. Lowenstein (1972b), in reflecting on this report, has commented that the small activity of pyruvate carboxylase in muscle may be an artifact of pyruvate carboxylase presence in fat cells within the muscle. Paul (1975) has noted and dis-

cussed the inherent technical difficulties of cleaning muscle samples of intercellular fat depots.

While it is generally conceded that propionyl-CoA carboxylase does occur in mammalian muscle tissues (Klotz et al., 1970), and that propionyl-CoA carboxylase favors methylmalonyl-CoA production in both liver and skeletal muscle (Utter 1969, Mayes 1971), the quantitative significance of this pathway for net CAC intermediate repletion in muscle may only be inferred. Since the pathway favors production of methylmalonyl-CoA and succinyl-CoA, adequate amounts of propionyl-CoA must be available for the combined action of propionyl-CoA carboxylase and methylmalonyl-CoA isomerase to be effective. One source of propionyl-CoA in mammalian tissues is from fatty acids of odd chain length which occur rarely in nature (Lehninger, 1970). In man, only 1 to 2% of the total fatty acids in stored lipids are of odd chain lengths (Bartley et al., 1968). On the other hand, in ruminant mammals, a large portion of the caloric intake of the animal may be accounted for by propionic acid formation by bacteria in the rumen (Bartley et al., 1968; Mayes, 1971). Propionyl-CoA can also arise from two other metabolic sources, the oxidative degradation of the free amino acids, valine and isoleucine. In this regard, one may not ignore recent evidence from Goldberg and Odessey (1972) and Beatty et al. (1974) who have proposed that the major locale for isoleucine, and valine catabolism in the rat is in skeletal muscle. In addition, Odessey et al. (1974) have suggested that in vivo metabolism of these amino acids by skeletal muscle may account for 14% of oxidative metabolism.

Phosphoenolpyruvate carboxykinase has been found in substantial amounts in liver, kidney and skeletal muscle (Opie and Newsholme, 1967). However, recent views hold that the major physiological function of this

enzyme in liver and skeletal muscle lies in the direction of decarboxylation of OAA with the formation of phosphoenolpyruvate as the important metabolic product (Rognstad and Katz, 1966; Gevers, 1967; Utter, 1969; Mayes, 1971). This pathway is considered an important link in the biosynthesis of glucose from pyruvate since the thermodynamics of the Embden-Meyerhoff pathway do not favor "uphill" conversion of pyruvate to phosphoenolpyruvate by pyruvate kinase.

Malic enzyme is also widely distributed in mammalian tissues (Utter, 1969). However, Molé et al. (1973) have reported that in rat skeletal muscle homogenates, the level of malic enzyme activity in the direction of malate production was quite low (0.69 μmole/g wet weight/min). Aerobic training enhanced this activity by only 50% and these authors concluded that this pathway was not of major concern in the alternative pathways of pyruvate metabolism. A major importance of this pathway for gluconeogenesis has been proposed (Utter, 1969; Lehninger, 1970) and it is generally accepted that this enzyme has limited activity in the direction of malate synthesis in muscle (Bowman, 1966; Davis et al., 1972).

The four enzymatic mechanisms discussed above are the only ones which have received serious consideration for CAC anaplerosis from two- or three-carbon sources in animal tissues. However, microorganisms and plant cells are known to possess three other CAC repletion pathways in addition to those noted above. These include the reactions catalyzed by phosphoenolpyruvate carboxylase (Bandurski and Greiner, 1953) (Equation 6), phosphoenolpyruvate carboxytransphosphorylase (Siu and Wood, 1962) (Equation 7) and malate synthetase (Wong and Ajl, 1956) (Equation 8).

(6) phosphoenolpyruvate + CO_2 + H_2O ⟶ oxaloacetate + P_i

(7) phosphoenolpyruvate + CO_2 + P_i $\rightleftharpoons$ oxaloacetate + PP_i

(8) acetyl-CoA + glyoxylate $\longrightarrow$ malate + CoA

The key enzymes of these latter 3 reactions have not yet been detected in animal tissues (Utter, 1969; Lehninger, 1970).

The apparent lack of major pathways in mammalian muscle for fixation of CO_2, as a mechanism for converting mono- to dicarboxylic acids, has led to the generally accepted theory that augmentation of muscle tissue contents of intermediates of the CAC occurs at the expense of the large pool of free amino acids in muscle tissues (Safer and Williamson, 1973). METABOLISM OF AMINO ACIDS TO CITRIC ACID CYCLE INTERMEDIATES IN MUSCLE TISSUES. The catabolic pathways of amino acid carbon skeletons to amphibolic intermediates of the CAC (Figure 1) in mammalian tissues are complex, with many multifunctional intermediates leading to branches and collateral routes. However, transamination (type reaction: Equation 9 and Figure 2) or oxidative deamination (3 type reactions: Equations 10, 11 and 12) generally constitute the initial stages in amino acid catabolism (Lehninger 1970; Mayes, 1971). These reactions are the mechanisms by which the α-amino groups are removed from the various amino acids.

(9) α-amino acid + αketoglutarate $\rightleftharpoons$ αketo acid + glutamate

(10) glutamate dehydrogenase

L-glutamate + NAD^+ $\rightleftharpoons$ αketoglutarate + NH_4^+ + NADH

(11) L-amino acid oxidase (E-FMN)

L-amino acid + H_2O + E-FMN $\rightleftharpoons$ αketo acid + NH_4^+ + E-$FMNH_2$

(12) D-amino acid oxidase (E-FAD)

D-amino acid + H_2O + E-FAD $\rightleftharpoons$ αketo acid + NH_4^+ + E-$FADH_2$

The reduced flavin nucleotides of both enzymes in equations (11) and (12) above are reoxidized directly by molecular oxygen (Equations 13 and 14)

(7) phosphoenolpyruvate + CO_2 + P_i $\rightleftharpoons$ oxaloacetate + PP_i

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(12) D-amino acid oxidase (E-FAD)

D-amino acid + H_2O + E-FAD $\rightleftharpoons$ αketo acid + NH_4^+ + $E\text{-}FADH_2$

The reduced flavin nucleotides of both enzymes in equations (11) and (12) above are reoxidized directly by molecular oxygen (Equations 13 and 14)

to produce peroxide which is subsequently decomposed to water and oxygen (Equation 15).

(13) $E\text{-}FMNH_2 + O_2 \longrightarrow E\text{-}FMN + H_2O_2$

(14) $E\text{-}FADH_2 + O_2 \longrightarrow E\text{-}FAD + H_2O_2$

(15) $2H_2O_2 \longrightarrow 2H_2O + O_2$

TRANSAMINATION. Studies of transaminase enzymes' distributions and activities in various mammalian tissues suggest that the catabolism of many of the dietary essential amino acids occurs exclusively in liver, whereas an important reaction in the catabolism of many of the nonessential amino acids is transamination in muscle (Figure 1).

Lin and Knox (1958) have reported that the enzymes associated with catabolism of the aromatic amino acids, phenylalanine and tryptophan, are confined to the liver of "rats". The transaminases for the branch chain amino acids, leucine, isoleucine and valine, are present in highest activity in kidney, followed by skeletal and cardiac muscle, and is in lowest concentration in liver cells of rats (Awapura and Seale, 1952; Ichihara and Koyama, 1966; Mimura et al., 1968).

The distribution of transaminases for nonessential amino acids is widespread in all mammalian tissues (Young, 1970). Liver and myocardium of rats are very high in activity of glutamate-oxaloacetate transaminase (Equation 16) (Laferte et al., 1963; Zimmerman et al., 1968; Howarth et al., 1968).

(16) glutamate + oxaloacetate $\rightleftharpoons$ αketoglutarate + aspartate

However, considerable glutamate-oxaloacetate transaminase is also found in skeletal muscle of all species of mammals which have been examined (Cohen, 1942), including man (Wrobelewski and LaDue, 1956; Klein and Chlond, 1967). In some mammals (i.e., rat, ox and horse), the activity

of this enzyme per gram of skeletal muscle tissue is equal to or greater than that found in liver (Freedland et al., 1965; Cardinet et al., 1967; Howarth et al., 1968). Glutamate-pyruvate transaminase (Equation 17) distribution in mammalian tissues generally exhibits a higher activity per gram of tissue in liver than in skeletal or cardiac muscle (Lafèrte et al., 1963; Zimmerman et al., 1968), although this pattern is reversed in the horse (Freedland et al., 1965), ox and pig (Cornelius et al., 1959).

(17) glutamate + pyruvate $\rightleftharpoons$ α ketoglutarate + alanine

Muscle constitutes approximately 45% of the tissue mass of the mammalian body, compared to 5% of the mass for liver (Lehninger, 1970). For those transaminases of high activity in muscle, the intensity of total transamination reactions therefore, far surpasses that associated with other tissues (Young, 1971). This can be correlated with studies of the degradation of amino acids in various tissues. Miller (1962) administered individual ^{14}C labelled amino acids to intact or hepatectomized rats and also to in vitro perfused liver. Hepatectomized animals were unable to convert the essential amino acids, arginine, lysine, threonine, methionine, histidine, phenylalanine and tryptophan to $^{14}CO_2$. However, hepatectomy had little effect on metabolism of the nonessential amino acids and also the branched chain amino acids, leucine, isoleucine and valine. These findings are substantially similar to reports by Aikawa et al. (1973) and Matsutaka et al. (1973) who studied inter-organ relationships in amino acid metabolism in intact or eviscerated-nephrectomized rats and in rat non-hepatic splanchnic organs, both in vivo and in vitro.

Odessey, Goldberg and coworkers (Goldberg and Odessey, 1972; Odessey and Goldberg, 1972; Odessey et al., 1974) have also validated these find-

ings with *in vitro* studies of various rat tissues. Goldberg and Odessey (1972) demonstrated that when ^{14}C labelled amino acids were presented individually to incubated rat diaphragms, $^{14}CO_2$ produced by the muscle accounted for 38% of the leucine, 36% of the isoleucine, 25% of the valine, 57% of the alanine, 47% of the glutamate and 53% of the aspartate taken up. Approximately 5% of the glycine, serine and proline incorporated by the diaphragms appeared as $^{14}CO_2$, while no $^{14}CO_2$ was produced by the other essential amino acids. This is quantitatively similar to work by Beatty *et al.* (1974) with red and white skeletal muscle tissues of rats. Alanine, leucine and glutamate catabolism contributed 20% of the total carbon dioxide production of both types of skeletal muscle and $^{14}CO_2$ production accounted for 17 to 51% of the uptake of these amino acids. Odessey and Goldberg (1972) have reported the metabolism of ^{14}C leucine to $^{14}CO_2$ in various tissues of rats. All tissues were highly active in this regard with their proportional activity being 1:1.14:3.86: 4.41;5.74 in liver, diaphragm, adipose tissue, brain and kidney, respectively.

TRANSAMINATIONS AND CITRIC ACID CYCLE REPLETION. The requirement of amino acid catabolism for net accumulation of CAC intermediates has been most clearly deomonstrated in perfused rat hearts. Metabolic interactions of amino acid catabolism with the CAC varies with the inclusion of different substrates in the perfusion mixture. Perfusion with glucose or carbohydrate sources alone (i.e., pyruvate) have resulted in large concentration increases in the CAC which are attributable to transamination reactions. Davis and Bremer (1973) have reported stoichiometric, reciprocal changes in alanine and glutamate accompanying 10 fold increases in the CAC of rat heart perfused with hyper-physiological pyruvate.

Safer and Williamson (1973) have found that net accumulation of CAC intermediates in glucose perfused rat hearts was directly related to double transaminations of glutamate-pyruvate and glutamate-oxaloacetate transaminases. However, Neely et al. (1972) have opposed these results. Glucose perfusion of rat hearts at 50 or 150 mmHg perfusion pressures resulted in no changes in CAC concentrations with the exception of a small malate decrease. Oxygen uptake was enhanced 2.5 fold by glucose addition as compared with a substrate free perfusion. However, while aspartate and glutamate were assessed in this study, alanine was not.

Perfusion of rat hearts with acetate, octanoate or the ketone bodies, β-hydroxybutarate and acetoacetate, alone or in combination with glucose resulted in a variety of patterns of CAC concentration responses and proposed feed-in mechanisms. Bowman (1966) found increased total CAC concentrations in rat hearts perfused with octanoate, acetate or β-hydroxybutarate. These changes were not ascribed to fixation of ^{14}C-bicarbonate to pyruvate. ^{14}C-aspartate however, was found to have a major effect on increased oxaloacetate and citrate concentrations. Randle et al. (1970) found that acetate or acetate plus glucose perfusion produced large changes in CAC contents while simultaneous increases in alanine and glutamate and decrease in aspartate were recorded. ^{14}C-acetate perfusion resulted in rapid isotope equilibration between αKG and glutamate but not between malate and aspartate. They concluded that glutamate-pyruvate transaminase is near equilibrium in rat heart while glutamate-oxaloacetate is slow in the direction of oxaloacetate production. The cause of the latter phenomenon was unexplained. Neely et al. (1972) examined glucose, glucose plus acetate and acetate perfusion of rat heart. In contrast to glucose and glucose plus acetate perfusion, where small CAC content changes

were noted, acetate alone resulted in marked increases in citrate, malate and glutamate, while aspartate decreased. These findings led to the conclusion that changes in the CAC associated with acetate perfusion, either alone or in addition to glucose, were not specifically concerned with regulation of CAC concentrations for optimal CAC flux, but were of utmost importance for regulation of glycolysis. The inhibitory effect of increased citrate concentration of phosphofructokinase was discussed.

Davis and Bremer (1973) have concluded that CAC intermediates are produced in 3 different reactions in rat heart under varied conditions of substrate availability (substrate free, pyruvate, acetate or acetoacetate). They suggest that alanine, glutamate and aspartate concentration changes are required for CAC content adjustments. However, CAC repletion was also partially under control of other pathways involving the branch chain amino acids. These pathways did not, however, require ammonia production and could occur in the face of decreased alanine and aspartate concentrations.

At an early stage in catabolism of most amino acids in skeletal and cardiac muscle the α-amino group is enzymatically removed by transamination to αKG or OAA. In such reactions, the amino group is transferred to the αketo acid substrate, leaving behind the corresponding αketo acid analog of the amino acid and causing amination of the αketo acid acceptor. For, αKG and OAA, these are glutamate and aspartate, respectively.

It is clear from an inspection of the carbon stoichiometry that a simple transamination between any amino acid and an αketo acid of the CAC cannot give rise to a net increase of cycle intermediates. While the carbon skeleton of the deaminated amino acid may be subsequently catabolized in the CAC, a dicarboxylic acid of the CAC provided a sub-

strate for the reaction to occur.

Subsequent transamination involving glutamate-oxaloacetate or glutamate-pyruvate transaminases may result in the collection of the amino groups of the various amino acids as the amino group of glutamate, alanine or aspartate. This sequence of possible transaminations has led to the proposal by Davis et al. (1972) and Safer and Williamson (1973) that coupled transaminations involving glutamate-pyruvate transaminase provide a major mechanism for replenishment of CAC intermediates in muscle. Davis et al. (1972) suggested that those amino acids which have corresponding high transaminase enzyme levels in muscle (i.e., leucine, isoleucine and valine) may also couple with glutamate-pyruvate transaminase and through subsequent reactions yield a net repletion of the CAC (Equations 18, 19 and 20).

(18) amino acid + α ketoglutarate $\rightleftharpoons$ CAC-carbon skeleton + glutamate

(19) glutamate + pyruvate $\rightleftharpoons$ α ketoglutarate + alanine

(20) net: amino acid + pyruvate $\rightleftharpoons$ CAC-carbon skeleton + alanine

Safer and Williamson (1973) suggested that based on evidence of reciprocal concentration changes of the amino acids glutamate, aspartate, alanine and CAC intermediates OAA, citrate, malate and αKG in rat hearts, a coupled effect of glutamate-oxaloacetate and glutamate-pyruvate transaminases is also supported (Equations 21, 22 and 23).

(21) aspartate + αketoglutarate $\rightleftharpoons$ glutamate + oxaloacetate

(22) glutamate + pyruvate $\rightleftharpoons$ α ketoglutarate + alanine

(23) net: aspartate + pyruvate $\rightleftharpoons$ oxaloacetate + alanine

However, it should be noted that in contrast to skeletal muscle where increased metabolic demands produce greater intramuscular concentrations

of aspartate and less free glutamate (Borst, 1962; Edington et al., 1973; Koziol and Edington, 1975), rat heart muscle demonstrates greater glutamate concentration and decreased aspartate concentration (Bowman, 1966; Randle et al., 1970; Davis and Bremer, 1973; Safer and Williamson, 1973). Since inferences based on concentrations alone can be misleading, little significance can be attached to this phenomena, though it would appear that the demonstration of coupled transaminations in rat heart as proposed by Safer and Williamson (1973) bears careful scrutiny in skeletal muscle.

Nonetheless, bearing the stoichiometry of transamination pathways in mind and considering the abundance of evidence for amino acid catabolism to CAC intermediates in skeletal muscle, these suggestions must be considered as a primary scheme for CAC repletion.

Evidence of a qualitatively similar nature to that mentioned above has been provided for CAC repletion from amino acid transaminations in insect flight muscle (Sacktor and Wormser-Shavit, 1966; Hansford and Sacktor, 1970; Hansford, 1975; Johnson and Hansford, 1975). However, the vast generic difference between mammals and insects contraindicates in depth comparison of precise metabolic interactions in the two genera and the above reports will not be enlarged upon here.

Similar evidence is notably lacking for skeletal muscle of higher vertebrates though inferences of such events may be derived from several lines of research. In general, increased metabolic activity is associated with increased concentrations of selected CAC intermediates in avian (Borst, 1962) and rat skeletal muscles (Edington et al., 1973; Koziol and Edington, 1975). However Graham et al. (1974) have noted decreases in OAA, succinate and malate between initiation and 7 min of exercise in

dog gastrocnemius muscle. None of these studies have reported the total balance of CAC intermediates in the muscle under study. However, the selected CAC intermediates reported for each muscle varied predictably with knowledge of fiber composition and duration of exercise. For instance, the fact that Graham et al. (1974) observed decreasing concentrations of OAA, succinate and malate in contracting dog skeletal muscle before 7 min, is supportive of evidence suggesting transient realignments of some CAC concentrations to support the malate-aspartate shuttle (Figure 3) during early exercise (Safer, 1975). Dog gastrocnemius muscle is known to produce large quantities of lactate during the initial 3 to 5 min of exercise but, thereafter, lactate formation declines steadily as exercise is prolonged (Hirche et al., 1971). Therefore, though Graham et al. (1974) did not report amino acid dynamics associated with observed trends in the CAC, this does not necessarily preclude simultaneous anaplerosis from amino acids (LaNoue and Williamson, 1971; Safer and Williamson, 1973; Safer, 1975).

Studies of the balance of amino acid metabolism in human skeletal muscle all support the preponderance of alanine output from muscle relative to all other amino acids (Carlsten et al., 1962; Felig and Wahren, 1971a, b; Ahlborg et al., 1974; Ellis et al., 1974). Felig and Wahren (1971a, b) have demonstrated that alanine efflux from human muscle accounts for 30 to 45% of the total nitrogen lost from the muscle at rest or during exercise. It was also shown that in mild, moderate and severe exercise, alanine output from leg muscles of men increased proportionally with the work done (55%, 90% and 500% above resting values respectively). More recently Ahlborg et al. (1974) have reported that men exercised for prolonged periods of time (4 hr), produced alanine from their leg muscles at a rate which increased with exercise duration. Alanine output was 3 fold

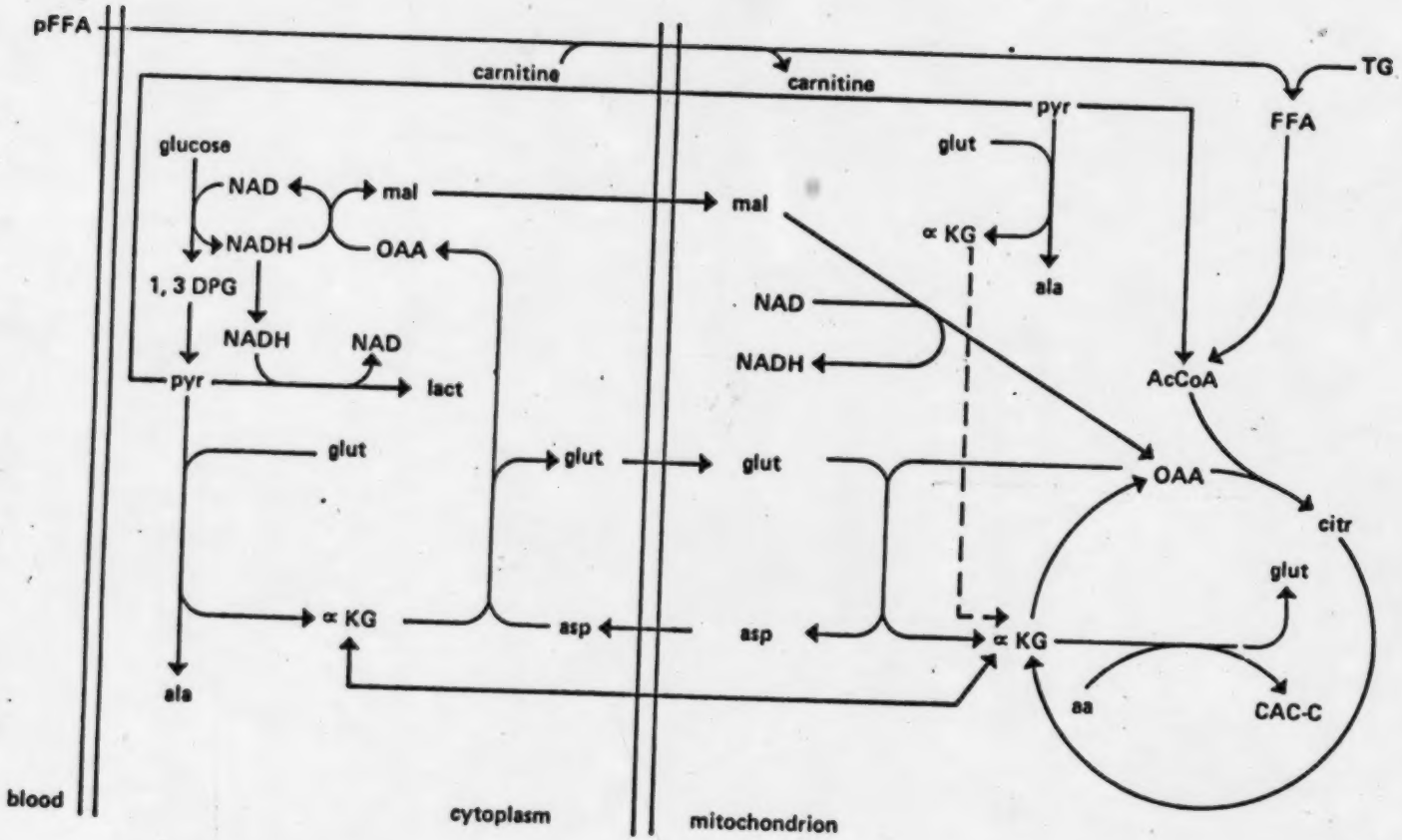


Figure 3 Metabolic flux scheme showing the relationship between glycolysis, the malate-aspartate shuttle, β-oxidation, amino acid oxidation and the citric acid cycle in skeletal muscle

over resting values after 4 hr of activity. The proportion of total amino acid balance accounted for by alanine in each of the above cases (Felig and Wahren, 1971a, b; Ahlborg et al., 1974) far exceeded the occurrence of alanine in muscle protein and thus de novo synthesis of alanine from glutamate-pyruvate transamination was suggested. This suggestion finds support in the work of Pozefsky and Tancredi (1972) where intrabrachial administration of hyper-physiological pyruvate to human forearm tissues resulted in marked outputs of alanine and uptake of the branched chain amino acids. Other amino acids were unaffected. It was suggested that with pyruvate as an amino group acceptor, facilitation of branch chain amino acid oxidation was achieved.

The efforts of Molé et al. (1974, 1975) also support glutamate-pyruvate transamination in human muscle. Molé et al. (1974) have concluded that alanine production of working human muscle is at least partially derived from transamination of pyruvate. Alanine output was shown to be highly related to the rate of glycolysis, lactate production and workload relative to the maximum aerobic capacity of untrained men. This report was further elaborated by comparisons of trained and untrained men during work at different percentages of their respective maximum aerobic capacity (Molé et al., 1975). Of particular interest in the latter study was the finding that for a given relative workload, the muscles of trained men oxidized more fats and less carbohydrate, produced less lactate, equal amounts of pyruvate and greater quantities of alanine than untrained men. Since glycolysis occurred at similar rates for a given relative workload in trained and untrained men, greater alanine production was therefore associated with greater oxidation of fats. The suggestion that greater net input of αKG from glutamate-pyruvate trans-

amination to the CAC in trained men was not discussed. However, it seems clear that adaptive responses in metabolism due to training may provide for greater repletion of the CAC through this transamination.

The appropriateness of the above authors' interpretations of metabolic events, based on amino acid balances in humans, has been verified in numerous studies of rat skeletal muscle tissues, in vitro (Goldberg and Odessey, 1972; Hider and Meade, 1972; Odessey and Goldberg, 1972; Ruderman and Lund, 1972; Beatty et al., 1974; Grubb and Snarr, 1974; Odessey et al., 1974), in situ (Aikawa et al., 1973; Matsutaka et al., 1973) and in vivo (Lefèbvre et al., 1972). These investigations have revealed, among other things, that alanine and glutamine are released in quantities far greater than would be expected on the basis of their content in muscle protein (Lefèbvre et al., 1972; Ruderman and Lund, 1972; Aikawa et al., 1973; Matsutaka et al., 1973). Further, provision of ^{14}C-carbohydrates to rat skeletal muscle results in a rapid production of ^{14}C-alanine (Hider and Meade, 1972; Grubb and Snarr, 1974; Odessey et al., 1974), thereby, indicating the function of glutamate-pyruvate transaminase in the direction of alanine and α KG production. This is consistent with the report that glutamate-pyruvate transamination is reversible in muscle with a Michaelis constant of this enzyme, for both glutamate and pyruvate, being within the range of their concentrations in muscle (Young, 1970). Therefore, perturbation of the concentrations of either of these substrates should enhance alanine synthesis.

Although some reference has already been made to the work of Odessey, Goldberg et al. in this review (pp.119), further elaboration of their reports provides substantial evidence in support of coupled transaminations in skeletal muscle. Skeletal muscle of rats metabolizes large

amounts of the branched chain amino acids, valine, isoleucine and leucine (Manchester, 1965; Goldberg and Odessey, 1972; Odessey and Goldberg, 1972). While the carbon chain of these amino acids was known to be degraded to CO_2 by muscle, the fate of the amino group was not clear until Odessey et al. (1974) reported that the amino groups released on oxidation of the branched chain amino acids, could account for all nitrogen recovered in alanine. Rat diaphragms were incubated in mediums containing glucose, with or without branched chain amino acids. The presence of branched chain amino acids consistently increased (by 50 to 100%) the release of alanine and glutamate. In contrast, other amino acids at twice their normal physiological concentrations, failed to affect alanine or glutamate production. A similar result was established with ammonia or adenine inclusion in the incubation medium, thereby demonstrating that amination of pyruvate was not occurring.

It is unlikely that the branched chain amino acids donate their amino groups directly to alanine. Odessey et al. (1974) have therefore suggested that the most likely mechanism for alanine production involves double transaminations of the type proposed by Davis et al. (1972) (Equations 18, 19 and 20).

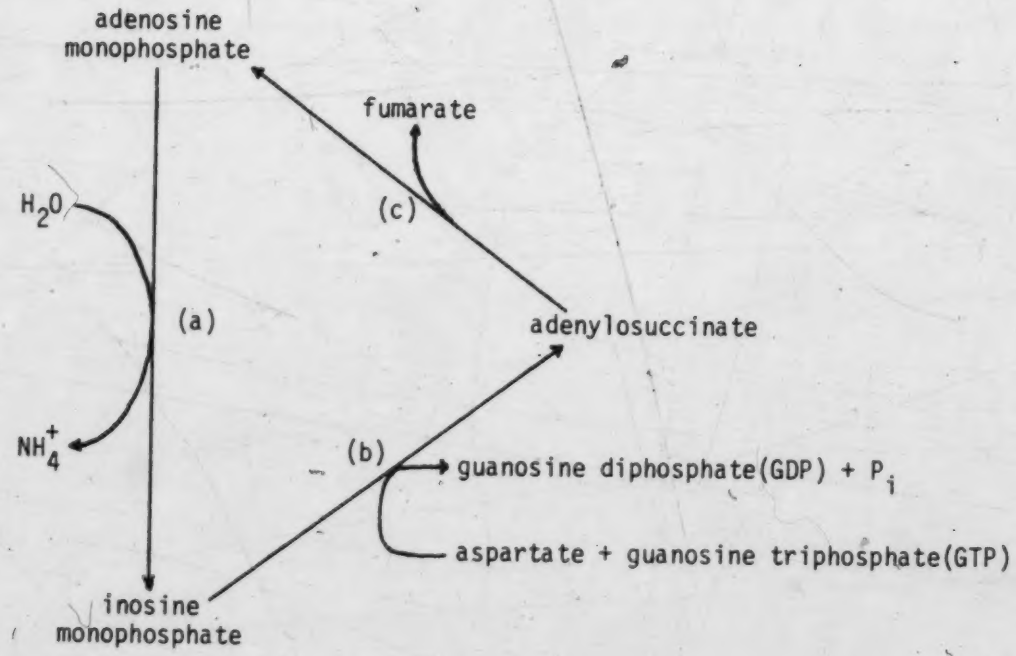
OXIDATIVE DEAMINATION. As stated earlier, the pattern of amino acid metabolism in mammals is one where the amino groups of the various amino acids are collected by the action of transaminases, thus ultimately appearing as the α-amino group of glutamate. Therefore, glutamate dehydrogenase (Equation 10) must be considered the key deaminase of mammalian tissues, particularly since glutamate is the only amino acid having a specific, highly active dehydrogenase (Lehninger, 1970). While L-amino oxidase and D-amino oxidase may be present in mammalian liver cells, they are of

very low concentration and only weakly active (Lehninger, 1970).

Comparison of organ distribution and activity of glutamate dehydrogenase has shown that this enzyme is of highest activity in liver, intermediate in kidney and brain, low in many other tissues and virtually absent in skeletal muscle (Lowenstein, 1972a, b). Adenylate deaminase (Equation 24) activity on the other hand is 60 fold higher in rat skeletal muscle than liver (Lowenstein, 1972a, b).

(24) adenosine monophosphate + H_2O ⟶ inosine monophophate + NH_4^+

The fact that ammonia production by skeletal muscle is proportional to the work performed (Parnas et al., 1927), has led Lowenstein and Tornheim (1971) and Lowenstein (1972a, b) to propose the purine nucleotide cycle (Figure 4) as an important source of amphibolic intermediates of the CAC. Strong evidence in support of this cycle was elaborated by these authors, however, quantitative analysis of the activity of this cycle in CAC repletion was not provided. Keul et al. (1972) felt that from the physiological point of view, the amino group donation by aspartate for reamination of inosine monophosphate back to adenosine monophosphate was much more important than a supply of dicarboxylic acids to the CAC. This was related to brief intense exercise where a rapid breakdown and resynthesis of ATP must occur. One rapid mechanism for regenerating ATP is catalized by myokinase, leading to AMP formation. AMP is then deaminated to inosine monophosphate by adenylate deaminase. Since ammonia is generated, the process is irreversible and reamination must occur by the subsequent steps of the cycle. Thus, the amino group of aspartate is used to rapidly reaminate inosine monophosphate and the fumarate produced may be further oxidized in the CAC.



Net: aspartate + GTP + H_2O ⟶ fumarate + GDP + P_i + NH_4^+

Figure 4 The purine nucleotide cycle (Lowenstein, 1972)
The enzymes in the cycle are :
(a) adenylate deaminase
(b) adenylosuccinate synthetase
(c) adenylosuccinase

APPENDIX II

NUTRITIVE CONTENTS OF BURGER BITS

TABLE 1 Nutritive Contents of Burger Bits (Standard Brands, Montreal)

Guaranteed Analysis

Crude Protein	Not less than 23%
Crude Fat	Not less than 7%
Crude Fiber	Not more than 5%
Ash	Not more than 10%
Moisture	Not more than 12%
Calcium	Min 1%, Max 2%
Salt (NaCl)	Min 1%, Max 1.8%

Ingredients

Ground corn, meat and bone meal, soybean meal, wheat middlings, animal fat - preserved with B.H.T., beef hydrolysate, dried beet pulp and/or tomato pomace, dried whey product, trace mineralized salt, soybean oil potassium chloride, iron oxide, vitamin B-12 supplement, vitamin A and E supplement, vitamin D supplement.

APPENDIX III

PHOTOGRAPHIC PLATES OF THE PREPARATION

Figure 1.

The surgical exposure of the gastrocnemius muscle revealing related blood vasculature and the truncated sciatic nerve

Figure 2.

The venous blood flow catheter in place and spatial fixation of the femur

Figure 3.

An arteriogram of the isolated, in situ gastrocnemius muscle.

Figure 4.

The mounted leg preparation revealing spatial fixation, the tension measurement system and the support of the mass of the partially severed foot

Figure 5.

The mounted leg preparation revealing placement of the perpetual saline drip, the stimulating electrodes and the needle-drain of the cavity underlying the gastrocnemius muscle

Figure 6.

The isolated calcaneus linked to the tension measurement complex

Figure 7.

The externalized venous blood flow system, the flow probe and the venous sampling valve

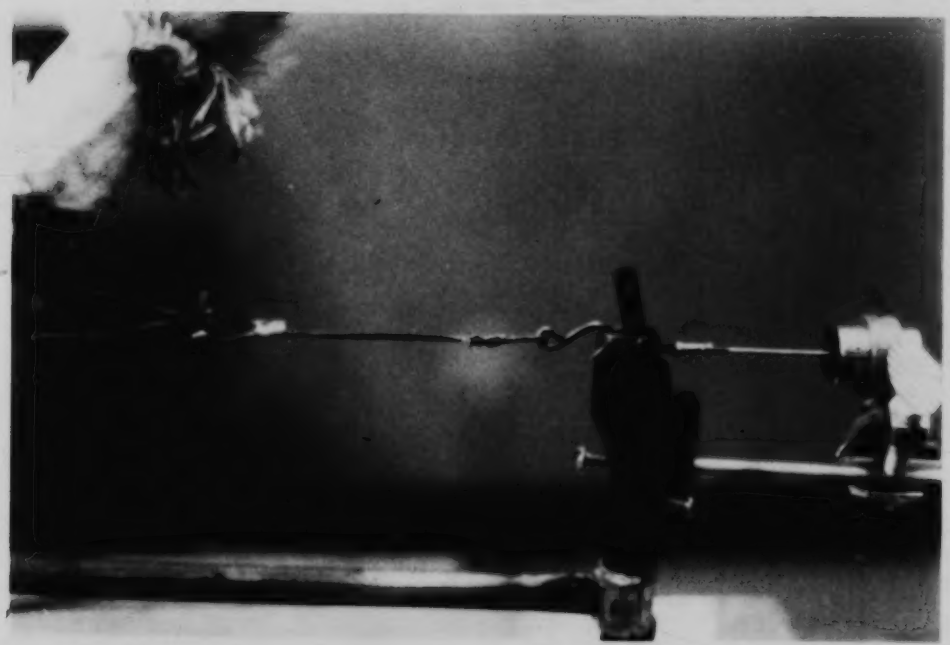
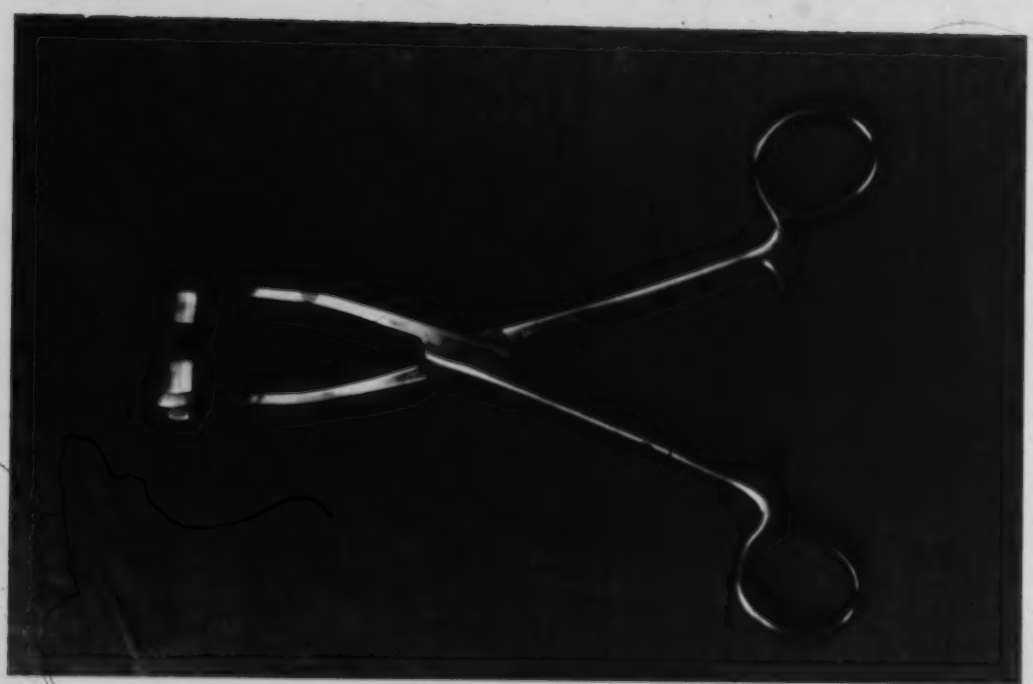


Figure 8.

The tension measurement system revealing the leaf-spring linked in series to the displacement transducer

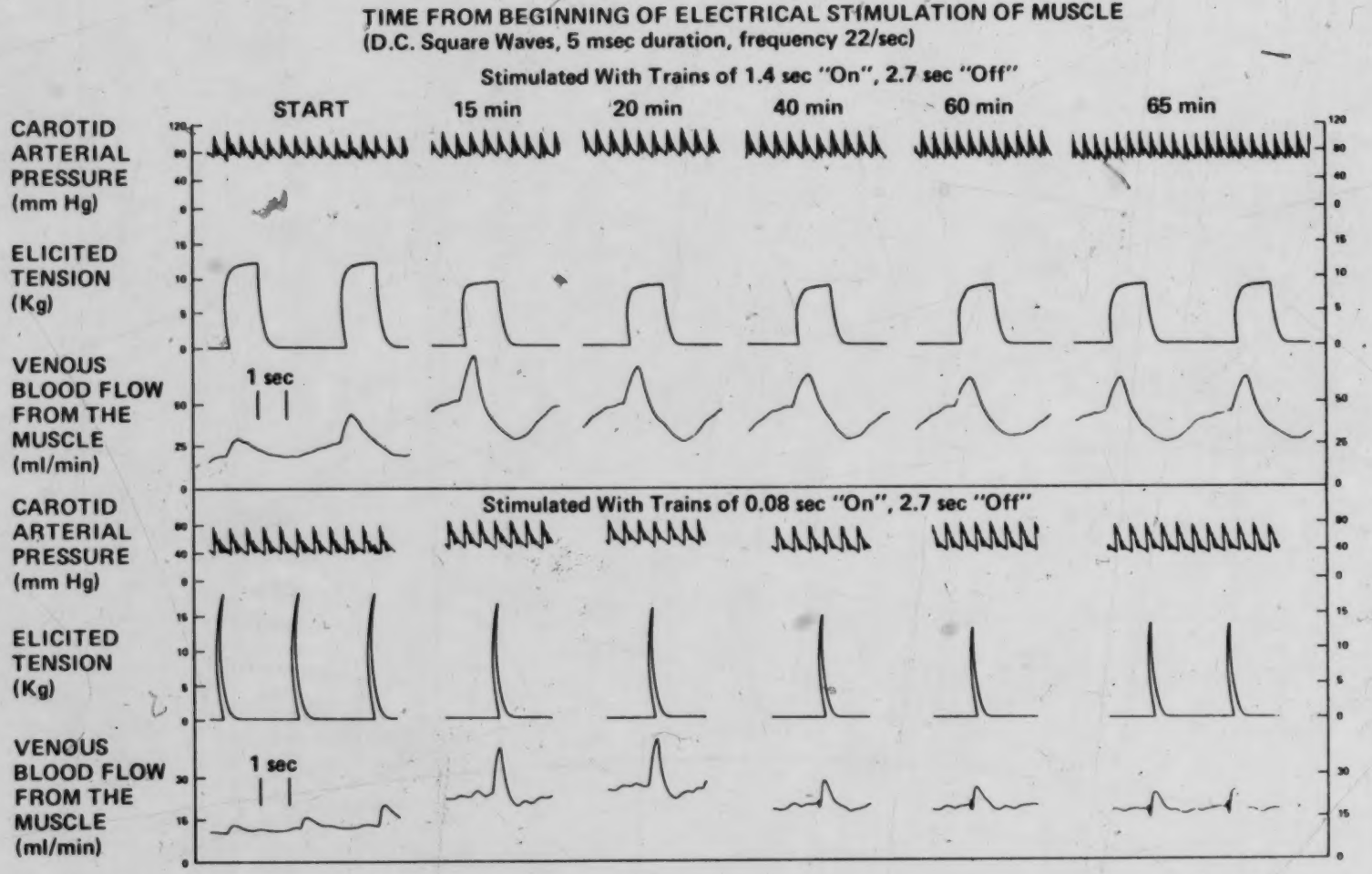
Figure 9.

The freeze clamp tongs, actual size 7 inches



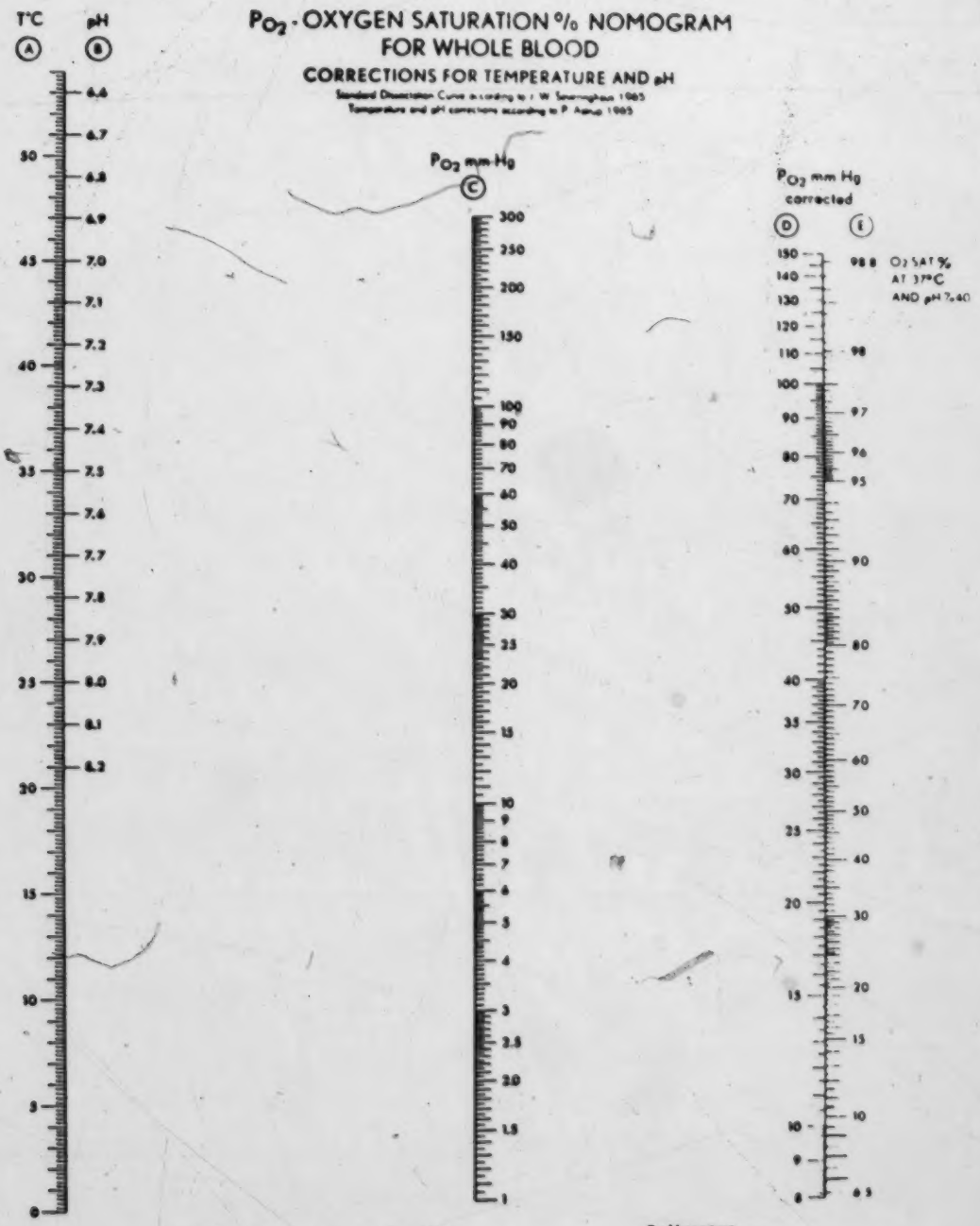
APPENDIX IV

REPRESENTATIVE DYNAGRAPH RECORDS


TIME FROM BEGINNING OF ELECTRICAL STIMULATION OF MUSCLE
(D.C. Square Waves, 5 msec duration, frequency 22/sec)
Stimulated With Trains of 1.4 sec "On", 2.7 sec "Off"
START
15 min
20 min
40 min
60 min
65 min
CAROTID ARTERIAL PRESSURE (mm Hg)
ELICITED TENSION (Kg)
VENOUS BLOOD FLOW FROM THE MUSCLE (ml/min)
1 sec
Stimulated With Trains of 0.08 sec "On", 2.7 sec "Off"
CAROTID ARTERIAL PRESSURE (mm Hg)
ELICITED TENSION (Kg)
VENOUS BLOOD FLOW FROM THE MUSCLE (ml/min)
1 sec

APPENDIX V

NOMOGRAM



P_{O_2} · OXYGEN SATURATION % NOMOGRAM FOR WHOLE BLOOD

CORRECTIONS FOR TEMPERATURE AND pH

Standard Dissociation Curve according to J. W. Severinghaus 1965
Temperature and pH corrections according to P. Astrup 1965

Clinical Diagnosis by Lab. Methods

Todd - Sanford Davidson & Henry page 667

Saunders - Philadelphia, London, Toronto

Radiometer

Scand. J.Clin. Lab. Invest.
17: 515, 1965.

APPENDIX VI

DESCRIPTION OF INDIVIDUAL DOGS

Dog No.	Age (yrs)	Sex	Weight (Kg)	Breed	Assigned Condition
42	1	F	23.2	Collie (X)	M20
43	1	M	22.9	Labrador (P)	M65
44	1	F	20.6	German Shep (P)	S20
45	1	F	17.6	German Shep (P)	M20
46	1	F	14.6	Labrador (P)	S65
47	7	M	22.4	German Shep (X)	M65
49	3	M	22.0	Collie (P)	M65
50	2	M	21.4	German Shep (P)	M65
51	1	M	15.3	Grey Hound (P)	S20
52	2	M	27.0	German Shep (P)	S65
53	3	F	20.4	Hound (X)	M65
54	1	M	19.9	German Shep (X)	M20
55	8	M	23.6	German Shep (X)	S65
57	1	F	20.0	Collie (X)	M65
58	2	M	23.4	German Shep (X)	S20
59	5	M	23.8	German Shep (X)	M20
60	3	M	20.5	Labrador (X)	M20
61	2	F	24.5	German Shep (X)	S65
62	2	M	23.0	German Shep (P)	S20
63	3	F	20.6	German Shep (X)	S20
64	1	F	27.0	German Shep (X)	S65
65	1	M	24.0	Samoyed (X)	S65
66	6	M	26.7	Golden Labrador (P)	M20
67	4	F	19.2	Beagle (P)	S20

TABLE 1 Age, sex, weight, breed and assigned experimental condition of individual dogs

* Predominent breed characteristic is noted; P = pure breed parentage, X = mixed breed parentage.

APPENDIX VII

PREPARATION VIABILITY DATA

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	20 min	40 min	60 min
Arterial [Glucose] (mg%)	M20	105.50 (4.78)	100.80 (3.96)			
	M65	113.20 (4.55)	107.70 (4.51)	110.70 (4.90)	109.70 (4.43)	107.80 (4.28)
	S20	106.50 (6.03)	105.00 (7.16)			
	S65	116.80 (6.76)	117.00 (7.59)	118.20 (8.04)	108.50 (7.07)	110.50 (8.94)
Arterial [Pyruvate] (mg%)	M20	0.65 (0.05)	0.58 (0.04)			
	M65	0.75 (0.11)	0.69 (0.10)	0.71 (0.05)	0.76 (0.10)	0.68 (0.06)
	S20	0.57 (0.15)	0.67 (0.11)			
	S65	0.68 (0.14)	0.64 (0.13)	0.73 (0.11)	0.71 (0.16)	0.66 (0.07)
Arterial [Lactate] (mg%)	M20	8.08 (1.11)	6.92 (0.82)			
	M65	10.50 (1.45)	10.40 (2.02)	10.00 (2.15)	9.91 (1.79)	10.25 (1.74)
	S20	12.75 (3.14)	13.00 (6.50)			
	S65	13.70 (3.70)	13.10 (3.18)	14.25 (4.01)	10.58 (2.29)	9.67 (2.03)
Arterial [Free Fatty Acids] (uEq/ml)	M20	0.98 (0.16)	0.82 (0.10)			
	M65	1.09 (0.20)	0.96 (0.17)	0.95 (0.22)	1.00 (0.29)	0.94 (0.25)
	S20	0.99 (0.15)	1.02 (0.15)			
	S65	1.33 (0.24)	1.45 (0.26)	1.25 (0.20)	1.15 (0.30)	1.00 (0.27)

TABLE 1 Arterial substrate and metabolite concentrations at the specified sample intervals: Mean (SEM), n=6.

PARAMETER	GROUP	REST	SAMPLE INTERVAL 15 min	60 min
Arterial [Alanine] (uM/ml)	M20	0.92 (0.10)	0.78 (0.13)	
	M65	0.92 (0.11)		0.89 (0.14)
	S20	1.24 (0.31)	0.97 (0.19)	
	S65	1.03 (0.16)		0.85 (0.15)
Arterial [Lysine] (uM/ml)	M20	0.15 (0.03)	0.12 (0.02)	
	M65	0.11 (0.02)		0.12 (0.02)
	S20	0.20 (0.06)	0.17 (0.04)	
	S65	0.13 (0.02)		0.11 (0.02)
Arterial [Glutamate] (uM/ml)	M20	0.06 (0.01)	0.06 (0.01)	
	M65	0.06 (0.01)		0.05 (0.005)
	S20	0.07 (0.01)	0.05 (0.01)	
	S65	0.06 (0.01)		0.05 (0.01)
Arterial [Aspartate] (uM/ml)	M20	0.013 (0.001)	0.011 (0.002)	
	M65	0.013 (0.002)		0.011 (0.001)
	S20	0.013 (0.001)	0.011 (0.002)	
	S65	0.016 (0.001)		0.012 (0.001)

TABLE 2 Arterial amino acid concentrations at the specified sample intervals: Mean (SEM), n=6.

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	20 min	40 min	60 min
Arterial Hemoglobin (gm %)	M20	14.22 (0.52)	13.88 (0.36)			
	M65	14.17 (0.68)	13.62 (0.77)	13.52 (0.71)	3.58 (0.79)	13.25 (1.00)
	S20	14.65 (0.95	14.12 (0.84)			
	S65	15.52 (0.97)	15.28 (1.00)	14.97 (0.89)	14.58 (0.84)	14.02 (0.89)
Arterial Hematocrit (%)	M20	43.67 (1.15)	42.00 (1.29)			
	M65	42.67 (2.00)	41.67 (2.00)	41.17 (1.91)	41.13 (2.08)	40.50 (2.82)
	S20	44.00 (2.71)	43.00 (3.16)			
	S65	47.33 (2.89)	46.50 (2.45)	45.50 (2.71)	44.00 (2.31)	42.50 (2.52)
Arterial Plasma Proteins (gm %)	M20	4.72 (0.22)	4.12 (0.20)			
	M65	4.72 (0.14)	4.33 (0.19)	4.30 (0.21)	4.10 (0.16)*	3.85 (0.26)*
	S20	4.73 (0.17)	4.35 (0.13)			
	S65	5.38 (0.26)	4.97 (0.28)	4.95 (0.30)	4.50 (0.26)	4.30 (0.33)

TABLE 3 Femoral arterial hemoglobin, hematocrit and plasma proteins at the specified sample intervals: Mean (SEM), n=6.

* significantly different from corresponding resting value ($p < 0.05$)

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	20 min	40 min	60 min
P_AO_2 (mmHg)	M20	99.25 (8.03)	100.35 (8.07)			
	M 65	96.63 (7.72)	99.70 (7.51)	97.75 (7.34)	98.88 (7.33)	98.05 (7.17)
	S20	84.50 (5.15)	88.25 (6.32)			
	S65	90.67 (8.87)	103.00 (7.19)	102.33 (7.08)	106.42 (8.04)	103.25 (7.57)
P_VO_2 (mmHg)	M20	40.55 (3.04	28.87 (2.05)			
	M65	42.22 (4.23)	30.47 (1.99)	30.28 (2.25)	32.13 (1.59)	33.03 (1.27)
	S20	44.53 (2.49)	24.50 (1.74)			
	S65	39.98 (2.25)	24.13 (1.82)	24.20 (1.41)	25.47 (1.65)	25.67 (1.31)
Arterial pH	M20	7.30 (0.02)	7.28 (0.02)			
	M65	7.29 (0.01)	7.29 (0.01)	7.28 (0.01)	7.25 (0.01)	7.24 (0.02)
	S20	7.30 (0.01)	7.28 (0.01)			
	S65	7.33 (0.01)	7.31 (0.01)	7.31 (0.01)	7.29 (0.01)*	7.29 (0.01)*

TABLE 4 Arterial and venous partial pressures of oxygen and arterial pH at the specified sample intervals: Mean (SEM), n=6

* significantly different from corresponding resting value ($p < 0.05$)

PARAMETER	GROUP	SAMPLE INTERVAL REST	15 min	20 min	40 min	60 min	65 min
Carotid Arterial Systolic Pressure (mmHg)	M20	88.83 (4.04)	87.33 (4.39)	85.17 (4.28)			
	M65	84.50 (4.43)	86.33 (2.94)	85.17 (3.56)	82.83 (4.16)	82.83 (4.16)	80.17 (4.16)
	S20	90.17 (1.29)	88.17 (2.08)	88.17 (2.23)			
	S65	93.50 (5.42)	92.50 (5.51)	92.00 (5.45)	90.83 (5.85)	90.67 (5.68)	89.00 (5.23)
Carotid Arterial Diastolic Pressure (mmHg)	M20	49.67 (4.79)	48.33 (4.58)	46.67 (4.86)			
	M65	46.33 (2.45)	46.17 (1.29)	47.83 (1.82)	44.00 (2.16)	44.33 (3.32)	43.17 (3.74)
	S20	50.83 (3.56)	50.00 (3.16)	47.83 (2.52)			
	S65	60.83 (4.54)	55.83 (5.51)	54.50 (5.00)	54.00 (4.86)	52.67 (9.60)	51.17 (4.93)

TABLE 5 Carotid arterial systolic and diastolic pressures at the specified sample intervals: Mean (SEM), n = 6.

PARAMETER	GROUP	SAMPLE INTERVAL					
		REST	15 min	20 min	40 min	60 min	65 min
Muscle Compartment Temperature (°C)	M20	38.97 (0.32)	38.85 (0.18)	38.83 (0.18)			
	M65	39.30 (0.18)	39.28 (0.26)	39.30 (0.26)	39.27 (0.18)	38.97 (0.26)	38.97 (0.26)
	S20	39.02 (0.18)	39.35 (0.18)	34.25 (0.18)			
	S65	39.63 (0.00)	39.53 (0.26)	39.63 (0.18)	39.60 (0.18)	39.32 (0.18)	39.33 (0.18)
Rectal Temperature (°C)	M20	38.97 (0.32)	38.80 (0.18)	38.77 (0.18)			
	M65	39.07 (0.26)	39.08 (0.18)	39.08 (0.18)	39.13 (0.00)	39.07 (0.18)	39.07 (0.18)
	S20	39.10 (0.18)	39.03 (0.26)	39.03 (0.26)			
	S65	39.33 (0.00)	39.40 (0.18)	39.40 (0.18)	39.35 (0.00)	39.18 (0.00)	39.17 (0.00)

TABLE 6 Muscle compartment and rectal temperatures at the specified sample intervals: Mean (SEM), n = 6.

APPENDIX VIII

HISTOCHEMICAL MICROGRAPHS

Figure 1

Micrographs of serial sections from right resting muscle (medial head) (Magnified 160X)

1. NADH Diaphorase

2. Myosin ATPase

3. Periodic Acid-Schiff

Figure 2

Micrographs of serial sections from left stimulated muscle of the M20 group (Magnified 160X)

1. Myosin ATPase

2. Periodic Acid-Schiff

Figure 3

Micrographs of serial sections from left stimulated muscle of the M65 group (Magnified 160X)

1. Myosin ATPase

2. Periodic Acid-Schiff

Figure 4

Micrographs of serial sections from left stimulated muscle of the S20 group (Magnified 160X)

1. Myosin ATPase

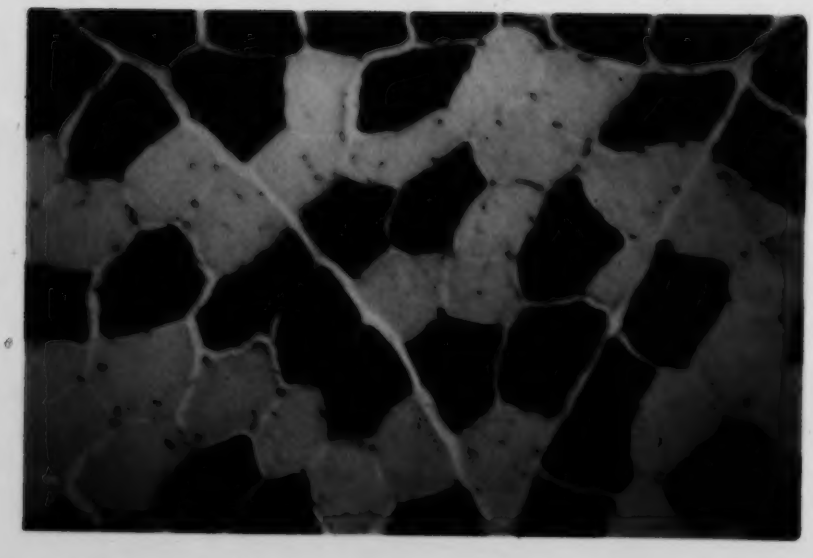
2. Periodic Acid-Schiff

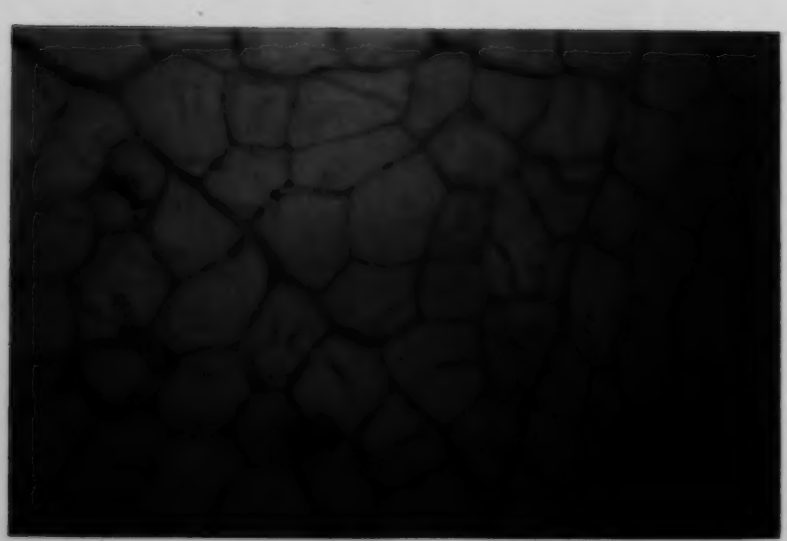
Figure 5

Micrographs of serial sections from left stimulated muscle of the S65 group (Magnified 160X)

1. Myosin ATPase

2. Periodic Acid-Schiff





APPENDIX IX

CORRELATION COEFFICIENTS

Number	Experimental Parameter
1	Oxygen uptake of the muscle
2	Muscle free aspartate concentration
3	Muscle free glutamate concentration
4	Muscle free alanine concentration
5	Muscle free lysine concentration
6	Lysine arterial-venous difference
7	Alanine arterial-venous difference
8	Glutamate arterial-venous difference
9	Aspartate arterial-venous difference
10	Pyruvate arterial-venous difference
11	Lactate arterial-venous difference
12	Glucose arterial-venous difference
13	Muscle blood flow
14	Carotid arterial systolic pressure
15	Carotid arterial diastolic pressure
16	Free fatty acids arterial-venous difference
17	Femoral arterial glucose concentration
18	Femoral arterial pyruvate concentration
19	Femoral arterial lactate concentration
20	Femoral arterial free fatty acids concentration
21	Femoral arterial alanine concentration
22	Femoral arterial lysine concentration
23	Femoral arterial aspartate concentration
24	Femoral arterial glutamate concentration
25	Femoral arterial hemoglobin concentration
26	Femoral arterial hematocrit concentration
27	Femoral plasma protein concentration

TABLE 1 Numerical designations of the correlated parameters found in Tables 2, 3, and 4.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	—													
2.	.46*	—												
3.	.28	.61*	—											
4.	.33	.33	.30	—										
5.	.53*	.25	.35	.36	—									
6.	.01	-.01	.07	.23	.22	—								
7.	.00	.05	.13	.03	.17	.82*	—							
8.	.45*	.05	.02	-.05	.31	-.05	-.06	—						
9.	.10	-.04	.05	.07	-.16	.15	.03	.37	—					
10.	.12	-.22	-.01	.55*	.40*	.18	.03	.21	.00	—				
11.	-01	.39	.06	-.09	-.11	-.04	.07	-.17	-.08	-.11	—			
12.	.16	.29	.21	.17	-.22	.31	.11	.16	.52*	-.10	.02	—		
13.	-.04	-.11	-.20	-.06	.03	-.10	-.25	.49*	.20	.23	-.00	.00	—	
14.	-.12	-.34	-.36	.03	-.09	.11	.05	.45*	.30	.24	-.33	.05	.31	—
15.	.26	-.38	-.41*	.01	.11	.28	.12	.54*	.23	.33	-.34	-.01	.45	.72

con't.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16.	-.09	-.04	-.03	.16	.05	-.05	-.10	.00	-.08	.30	.25	.05	.11	.06
17.	-.08	-.45*	-.50*	.04	-.28	.25	.11	.23	.26	.35	-.14	.25	.31	.48*
18.	.36	-.10	-.10	.45*	.47*	.32	.18	.33	.13	.70*	-.21	.03	.19	.40*
19.	.23	-.28	-.42*	.29	.13	.45*	.32	.16	.19	.28	-.04	.02	.02	.43*
20.	-.07	-.11	.14	.13	.09	.35	.19	-.10	.17	.11	-.43*	.19	.04	-.03
21.	.28	.07	-.01	.59*	.53*	.53*	.40*	-.04	-.18	.40*	-.04	-.07	-.04	.13
22.	.39	.15	.16	.43*	.72*	.47*	.40*	-.07	-.31	.26	-.19	-.20	-.12	-.02
23.	.29	.09	.11	.36	.04	.12	-.03	.17	.42*	.05	-.14	.26	-.15	.07
24.	.31	.19	.19	.31	.34	.19	.13	.23	-.19	.29	.07	-.15	.18	-.09
25.	-.03	-.30	-.09	.19	.15	.35	.13	-.30	-.07	.23	-.49*	.04	-.16	.11
26.	-.13	-.43*	-.13	.09	-.01	.27	.08	-.30	.02	.23	-.51*	.08	-.12	.13
27.	.27	-.15	-.44*	-.02	.03	.15	-.00	.45*	.07	.15	-.18	.20	.39	.36

con't.

	15	16	17	18	19	20	21	22	23	24	25	26	27
16.	.04	—											
17.	.49*	-.02	—										
18.	.39	.17	.52*	—									
19.	.44*	-.11	.56*	.47*	—								
20.	.08	-.05	-.21	.03	-.12	—							
21.	.20	-.04	.10	.43*	.62*	.04	—						
22.	.08	-.02	-.22	.32	.32	.28	.84*	—					
23.	.07	-.36	.23	.19	.40*	-.14	.33	.05	—				
24.	.23	-.07	.20	.24	.31	-.32	.49*	.30	.37	—			
25.	.35	-.08	.12	.14	.17	.34	.28	.35	-.04	.06	—		
26.	.32	-.01	.18	.08	.13	.37	.17	.22	-.02	.02	.95*	—	
27.	.65*	.15	.53*	.45*	.31	-.06	.15	.02	.17	.36	.14	.15	—

TABLE 2 Correlation coefficients among selected experimental parameters under resting conditions (n=24)

* significant correlation ($p < 0.05$)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	—													
2.	.01	—												
3.	-.58	.27	—											
4.	.55	-.49	-.64*	—										
5.	-.16	.06	.31	-.07	—									
6.	-.07	.21	.16	-.30	-.30	—								
7.	-.24	.29	.20	-.42	-.34	.91*	—							
8.	-.54	.21	.53	-.25	.19	-.42	-.31	—						
9.	-.62*	.13	.44	-.48	.17	.08	.05	.62*	—					
10.	-.80*	.22	.68*	-.56	.47	.04	.28	.48	.36	—				
11.	-.45	.24	.82*	-.59*	.23	-.14	-.09	.72*	.63*	.45	—			
12.	.02	-.33	-.26	.43	.51	-.54	-.59*	.21	.16	.07	-.08	—		
13.	.75*	.09	.23	.21	-.31	.06	-.11	-.22	-.12	-.70*	.02	-.07	—	
14.	-.30	-.16	.10	.23	-.43	-.15	-.11	.35	-.12	.05	.02	-.19	-.34	—
15.	-.07	-.61*	.21	.44	-.11	-.03	-.14	.02	-.15	-.08	.05	.00	-.02	.57*

con't.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16.	.28	.38	-.17	-.18	-.27	-.02	-.05	.07	.03	-.40	-.03	-.43	.30	-.10
17.	.14	-.61*	-.02	.55	.08	-.06	-.28	-.03	.04	-.25	.04	.35	.08	.12
18.	-.04	-.20	-.13	.55	.30	-.61*	-.59*	.46	-.06	.14	-.11	.69*	-.24	.39
19.	.26	-.58*	-.53	.81*	.19	-.55	-.69*	-.11	-.29	-.39	-.48	.59*	-.12	.27
20.	.16	-.16	-.27	.21	.00	-.38	-.29	-.01	-.30	-.23	-.28	-.21	-.06	.12
21.	.29	-.05	-.35	.45	.60*	-.48	-.49	-.15	-.39	-.07	-.47	.44	-.18	-.15
22.	.34	-.24	-.39	.42	.56	-.55	-.50	-.30	-.54	-.10	-.47	.37	-.14	-.23
23.	.09	-.15	-.04	.09	.54	-.10	-.31	-.10	.27	-.18	-.08	.32	.10	-.43
24.	-.26	-.20	.53	-.02	.32	.23	.05	.10	.26	.17	.22	-.03	-.10	.10
25.	.19	-.32	-.04	.40	.39	-.00	-.22	-.36	-.26	-.21	-.30	.05	-.17	.02
26.	.15	-.39	-.06	.46	.42	-.05	-.23	-.35	-.30	-.14	-.32	.15	-.27	.04
27.	.18	-.20	-.18	.36	-.16	.28	.39	-.30	-.47	.02	-.36	-.21	-.12	.07

con't.

	15	16	17	18	19	20	21	22	23	24	25	26	27
16.	-.45	—											
17.	.60*	-.43	—										
18.	.28	-.22	.18	—									
19.	.40	-.19	.51	.64*	—								
20.	.00	.62*	-.24	.16	.27	—							
21.	-.06	.07	-.07	.54	.63*	.50	—						
22.	-.03	-.00	-.05	.38	.58*	.56	.91*	—					
23.	.03	.14	.10	.09	.35	.22	.54	.41	—				
24.	.61	-.17	.30	.09	.10	.00	.12	-.02	.59*	—			
25.	.44	-.11	.50	.09	.59*	.21	.54	.47	.57	.59*	—		
26.	.43	-.23	.59	.16	.64*	.17	.54	.50	.44	.49	.97*	—	
27.	.15	-.12	.27	-.01	-.00	.18	-.00	.10	-.45	-.16	.09	.23	—

TABLE 3 Correlation coefficients among selected experimental parameters after 15 minutes of muscle stimulation (n=12)

* significant correlation ($p < 0.05$)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	—													
2.	.13	—												
3.	-.63*	.18	—											
4.	-.12	-.64*	-.21	—										
5.	-.35	.28	.42	-.12	—									
6.	-.28	-.06	-.17	.06	-.24	—								
7.	.05	.27	.12	-.16	.22	.39	—							
8.	-.12	-.28	.13	.16	.11	-.24	-.05	—						
9.	-.21	.32	.31	-.39	.15	.61*	.63*	.13	—					
10.	-.68*	.16	.51	.20	.35	.16	.01	-.36	-.00	—				
11.	-.31	-.10	.48	.17	.18	-.05	-.28	.37	.23	.24	—			
12.	.50	.02	-.57*	.28	-.36	-.01	-.18	-.41	-.43	-.03	-.04	—		
13.	.75*	.15	-.69*	-.18	-.61*	.02	-.30	-.45	-.25	-.44	-.29	.59*	—	
14.	.07	.07	-.40	-.22	-.33	.76*	.39	-.00	.64*	-.23	-.20	-.01	.23	—
15.	.20	.53	-.38	-.43	-.05	.56*	.57*	-.25	.53	-.10	-.51	.04	.26	.79

con't.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16.	.05	.17	-.00	-.32	-.25	.20	.45	.40	.45	-.41	-.19	-.10	-.12	.49
17.	-.05	-.18	-.17	.05	-.20	.53	.15	.53	.64*	-.30	.32	-.19	-.05	.75*
18.	-.12	-.28	-.04	-.02	-.03	.39	.20	.57*	.59*	-.25	.22	-.52	-.21	.62*
19.	-.08	-.45	.06	.42	-.01	.09	-.30	.31	.09	-.09	.81*	.16	-.06	-.10
20.	-.13	.42	.08	-.18	.12	.02	.40	.30	.15	.13	-.18	-.02	-.31	.14
21.	-.09	.19	.10	.43	.18	-.05	-.18	.19	.03	.38	.63*	.27	-.09	-.11
22.	-.06	.47	.23	-.02	.76*	-.29	.23	.29	.17	.27	.34	-.01	-.41	-.19
23.	.32	.08	-.24	-.19	-.44	.17	-.22	.21	.28	-.44	.13	-.20	.49	.40
24.	.55	-.16	-.16	-.00	-.30	-.70*	-.33	.55	-.37	-.63*	.06	.03	.23	-.26
25.	.03	.11	.00	.02	.39	.02	.55	-.02	.15	.31	-.02	.20	-.30	.14
26.	.05	.11	.02	-.00	.37	.01	.56*	-.03	.16	.29	-.01	.20	-.29	.13
27.	.40	.17	-.27	-.07	-.00	.14	.40	.41	.53	-.46	-.00	-.11	.09	.58*

con't.

	15	16	17	18	19	20	21	22	23	24	25	26	27
16.	.43	—											
17.	.32	.43	—										
18.	.25	.24	.86*	—									
19.	-.52	-.21	.40	.22	—								
20.	.40	.61*	.03	-.02	-.44	—							
21.	-.15	-.25	.23	.01	.44	.03	—						
22.	.07	-.04	.00	.00	.06	.41	.60*	—					
23.	.20	.01	.54	.54	.14	-.23	.11	-.24	—				
24.	-.36	.18	.05	.09	.07	-.00	.01	-.01	.34	—			
25.	.28	.08	-.00	.08	-.16	.41	.11	.54	-.50	-.18	—		
26,	.27	.10	-.00	.08	-.16	.41	.08	.52	-.50	-.16	.99*	—	
27.	.49	.41	.70*	.61*	.03	.07	.27	.30	.47	.28	.17	.16	—

TABLE 4 Correlation coefficients among selected experimental parameters after 60 minutes of muscle stimulation (n=12)

* significant correlation ($p < 0.05$).